"Flatter me, and I may not believe you. Criticize me, and I may not like you. Ignore me, and I may not forgive you. Encourage me, and I will not forget you."

- William Arthur Ward

University of Alberta

Bacteriophage therapy for controlling *Escherichia coli* O157:H7 in feedlot cattle

by

Erica Ann Rozema

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DEDICATION

I would like to dedicate this thesis:

1) to James for all of the time we spent apart. You kept me going.

2) and to my parents who offered to pay for one degree but ended up paying for two.

ABSTRACT

Oral and rectal administration of bacteriophage was compared for mitigating the fecal shedding of *Escherichia coli* O157:H7 by inoculated steers. Shedding of nalidixic acid-resistant (Nal^R) *E. coli* O157:H7 was monitored over 83 D following oral (3.3×10^{11} pfu), rectal (1.5×10^{11} pfu), both oral and rectal (4.8×10^{11} pfu) or no treatment with an O157-specific bacteriophage cocktail. Orally treated steers presented the fewest ($P = 0.06$) Nal^R *E. coli* O157:H7 culture-positive samples compared to rectally and orally + rectally treated steers. This may be attributed to higher ($P = 0.03$) mean bacteriophage shedding levels (log PFU/g of feces) in orally treated than in rectally treated steers. Control animals obtained bacteriophage *via* the environment, and shed at levels similar to that of REC ($P = 0.39$). Oral administration of bacteriophage via the feedlot environment may be an efficacious large-scale mitigation strategy for *E. coli* O157:H7 in cattle.

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ABBREVIATIONS

bp	base pairs
cc	cubic centimeters
CFU	colony-forming unit
CT-SMAC	sorbitol MacConkey agar with cefixime and potassium tellurite
CT-SMACnal	CT-SMAC with nalidixic acid
D	day
DNA	deoxyribonucleic acid
ds	double-stranded
EHEC	Enterohemorrhagic *Escherichia coli*
FYM	farmyard manure
GIT	Gastrointestinal tract
gp	gene product
g	grams
h	hours
HMC	hydroxymethylcytosine
HUS	Hemolytic uremic syndrome
IMS	immunomagnetic separation
L	litre
µg	microgram
µl	microliter
µm	micrometer
mg	milligram

ml	milliliter
min	minutes
MNA	modified nutrient agar
mTSB	modified tryptic soy broth
mTSB+n	modified tryptic soy broth with nalidixic acid
MOI	multiplicity of infection
nM	nano molar
Nal^R	nalidixic acid resistant
PBS	phosphate buffered saline
PFU	plaque-forming unit
PFGE	pulsed field gel electrophoresis
RNA	ribonucleic acid
s	seconds
ss	single-stranded
STEC	Shiga toxin-producing *Escherichia coli*
TEM	Transmission Electron Microscopy
TSB	tryptic soy broth
VTEC	Verotoxigenic *Escherichia coli*
VFA	Volatile fatty acid
w/v	weight per volume

1. GENERAL INTRODUCTION

1.1 Introduction

Escherichia coli O157:H7, the predominant serotype of the enterohemorrhagic *E. coli* (EHEC) subgroup of Shiga toxin-producing *E. coli* (STEC), also know as verotoxigenic *E. coli* (VTEC), is an important pathogen with respect to foodborne illness in Canada. In 2006, the annual incidence rate of *E. coli* O157:H7-related disease was 2.9/100,000 (Public Health Agency of Canada, 2007) This number, however, greatly underestimates the actual incidence of illness as only a quarter of cases are typically reported (Majowicz et al., 2005; Michel et al, 2000). The number of VTEC-related cases decreased from 1997 to 2004 (Public Health Agency of Canada, 2007). However, in 2005 the number rose sharply, and sustained similar levels in 2006 (Public Health Agency of Canada, 2007). Symptoms of infection include watery diarrhea, which can develop into hemorrhagic colitis (Riley et al., 1983). Though patients typically recover from illness, a number develop hemolytic uremic syndrome (HUS), a potentially fatal disease of the kidneys (Goldwater, 2007).

There are various modes of transmission of this pathogen to humans, including person-to-person (Mannix et al., 2007; Reida et al., 1994), animal-to-person (Crump et al., 2002) and consumption of contaminated food and water (Cieslak et al., 1993; Sewell and Farber, 2001). Between 1982 and 2002, 41% and 6% of the foodborne *E. coli* O157:H7 outbreaks in the United States were associated with ground beef and other beef products, respectively (Rangel et al., 2005).

Ruminants, particularly cattle, have been identified as a significant reservoir of *E. coli* O157:H7 (Hancock et al., 2001; Rasmussen and Casey, 2001; Renter and Sargeant, 2002) and the contamination of hides and the fecal shedding of *E. coli* O157:H7 by cattle has been correlated with adulteration of beef products (Elder et al., 2000). For these reasons a great deal of effort has been put into the investigation of methods to control *E. coli* O157:H7 in cattle.

Mitigation strategies have included minimizing contact between cattle and the pathogen (farm management), alteration of the gastrointestinal microbes (diet and probiotics), and direct attack methods (immunization and bacteriophage therapy). The use of bacteriophages to treat or prevent infection is not a new concept, as it dates back to the early 1900's. Indeed, researchers in previous studies have used this strategy to eliminate *E. coli* O157:H7 from mice (Tanji et al., 2005; Sheng et al., 2006) and calves (Waddell et al., 2000). However, very few studies have investigated the application of *E. coli* O157-specific bacteriophages to mitigate *E. coli* O157:H7 in adult ruminants (Bach et al., 2003; Sheng et al., 2006).

1.2 Objectives

The objectives of this research were to assess the use of bacteriophage therapy in mitigating the fecal shedding of *Escherichia coli* O157:H7 in feedlot cattle and to monitor experimental *E. coli* O157:H7 and O157-specific bacteriophages in the animal environment. A mixture of O157-specific bacteriophages rV5, wV7, wV8, and wV11 were orally, rectally or administered by both routes and assessed for their ability to decrease the fecal shedding of *E. coli* O157:H7 in orally inoculated feedlot cattle.

Water, feed, and pen-floor manure samples were analyzed for both *E. coli* O157:H7 and O157-specific bacteriophages to identify possible bacteriophage vectors. *E. coli* O157:H7 isolates collected from the beginning and from the end of the 83 D trial were analyzed by pulsed-field gel electrophoresis to determine if certain strains persisted more than others or developed resistance to bacteriophage over the course of the trial. Strains that exhibited slightly altered PFGE patterns were assessed for their susceptibility to the experimental bacteriophage used for therapeutic treatment.

1.3 Relevance of Research

At this time there is no known study examining the effect of bacteriophage therapy on adult cattle in a feedlot environment. Both bacteriophage and *E. coli* O157:H7 have been isolated from environmental samples and it is thought that cattle can acquire *E. coli* O157:H7 and bacteriophage from the environment. Characterization of the ecological relationship between bacteriophage and their target host could result in the development of more efficacious bacteriophage administration strategies for employment in commercial feedlots.

The only other known O157-specific bacteriophage study in cattle administered bacteriophage both orally and rectally (Sheng et al., 2006). This study was actually published shortly after the present work was completed. The present research compares oral and rectal administration of bacteriophage in establishing bacteriophage populations within feedlot cattle as well as the effectiveness of oral and rectal administration in mitigating the fecal shedding of *E. coli* O157:H7.

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Chapter 2

2. LITERATURE REVIEW

2.1 *Escherichia coli* O157:H7

2.1.1. Identification as a human pathogen

Escherichia coli O157:H7 was first identified as a human pathogen following two outbreaks of suspected foodborne illness in the United States in 1982 (Riley et al., 1983; Wells et al., 1983). Patients presented with similar symptoms, including severe abdominal cramping and watery diarrhea, followed by bloody diarrhea. *E. coli* O157:H7, a rarely documented serotype at the time, was isolated from 9 of 12 stool samples collected. Upon further investigation, an association between outbreaks and eating at a particular fast-food chain was found. *E. coli* O157:H7 was then also isolated from a beef patty from a suspected lot of meat which lead investigators to concluded that the pathogen was transmitted by undercooked meat (Riley et al., 1983).

Escherichia coli O157:H7 is the most dominant disease-causing serotype in the EHEC group (Coia, 1998; Hockin and Lior, 1987; Karmali, 1989; Smith and Scotland, 1988). *E. coli* serotypes included in this group are characterized by causing hemorrhagic colitis and hemolytic uremic syndrome (HUS) (Levine, 1988; Nataro and Kaper, 1998). Serotypes in this category also express high levels of Shiga toxins (or verotoxins, verocytotoxins) (Levine and Vial, 1988, O'Brien and Holmes, 1987; O'Brien and Kaper, 1998; Paton and Paton; 1998), which are involved in the pathogenesis of these bacteria.

2.1.2. Modes of Transmission

A recent study examined the epidemiology of *E. coli* O157:H7 outbreaks that occurred in the United States between 1982 and 2002 (Rangel et al., 2005). The modes of transmission that have been associated with human illness include foodborne (see references below), person-to-person (Belongia et al., 1993), waterborne (Friedman et al., 1999; Licence et al., 2001; Swerdlow et al., 1992; Verma et al., 2007), animal contact (Ogden et al., 2002) and laboratory-related, with foodborne transmission being associated with 52% of the recorded outbreaks (Rangel et al., 2005). A large number (21%) of outbreaks reported no identified source (Panaro et al., 1990; Rangel et al., 2005).

Foodborne outbreaks have been attributed to the consumption of produce (Ackers et al., 1998; Cieslak et al., 1997; Hilborn et al., 1999), dairy products (Clark et al., 1997; Espie et al., 2006; Goh et al., 2002; Liptakova et al., 2004; McIntyre et al., 2002), juices (Besser et al., 1993; Cody et al., 1999) and beef/meat products (Bell et al., 1994; Belongia et al., 1991; Riley et al., 1983; Stevenson and Hanson, 1996; Willshaw et al., 1994). According to Rangel et al., (2005), 47% of outbreaks between 1982 and 2002 were associated with the consumption of ground beef or other beef products.

2.1.3. Cattle as a primary reservoir of *E. coli* O157:H7

Ruminants, particularly cattle, are considered to be a primary reservoir of *E. coli* O157:H7 (Hancock et al., 2001; Rasmussen and Casey, 2001; Renter and Sargeant, 2002). Illness associated with *E. coli* O157:H7 [illegible] linked to the consumption of beef and dairy products (Bell et al., 1994; Borczyk et al., 1987; Buchanan and Doyle, 1997; Currie et al., 2007; Griffin and Tauxe, 1991; Honish et al., 2007; Jay et al., 2004; Laine et al., 2005 Martin et al., 1986; Maruzumi et al., 2005; Ostroff et al., 1990; Padhye and

Doyle, 1992; Riley et al., 1983; Wells et al., 1983, and Willshaw et al., 1994), food and water that has been contaminated with bovine feces (Armstrong et al., 1996; Besser et al., 1993; Borczyk et al., 1987; Chapman et al., 1993; Chapman et al., 1997; Doyle, 1991; Hancock et al., 1994; Mead and Griffin, 1998), or contact with ruminant animals (Crump et al., 2003, Renwick et al., 1993; Rice et al., 1995; Synge et al., 1993). It has also been found that the incidence of human illness tends to coincide with the seasonal shedding of *E. coli* O157:H7 by cattle (Hancock et al., 1997; Ostroff et al., 1989; Martin et al., 1990)

Fecal prevalence of *E. coli* O157:H7 in individual cattle has been shown to range from 0% to 18.8% and from 0% to 95.5% among herds or feedlots, respectively (Callaway et al., 2006; Callaway et al., 2004; Cho et al., 2006; Dargatz et al., 1997; Dunn et al., 2004; Eriksson et al., 2005; Ezawa et al., 2004; Fegan et al., 2004; Keen and Elder, 2000; Khaitsa et al., 2006; Renter et al., 2004; Riley et al., 2003; Sargeant et al., 2004; Schouten et al., 2004; Stanford et al., 2005; Vicente et al., 2005). However, it is difficult to truly estimate the prevalence of *E. coli* O157:H7 in a herd or cattle population at any given time for a number of reasons. Cattle harboring the pathogen are apparently healthy, showing no clinical signs of disease (Borczyk et al., 1987; Chapman et al., 1993; Chapman et al., 1997; Hancock et al., 1994; Mechie et al., 1997; Montenegro et al., 1990; Wells et al., 1991; Zhao et al., 1995). Also, the fecal shedding of *E. coli* O157:H7 appears to be transient and seasonal, with shedding periods ranging from days to months and peak prevalence occurring between April and September (Besser et al., 1997; Besser and Hancock, 1994; Chapman et al., 1997; Hancock et al., 1997; Hancock et al., 1994; Lynn et al., 1998; Mechie et al., 1997; Rahn et al., 1997; Sanderson et al., 1999; Van Donkersgoed et al., 2001; Wray et al., 2000). Finally, it is felt that commonly used

methods to determine the prevalence of *E. coli* O157:H7, such as isolation and culturing from feces, underestimates the actual herd prevalence (Keen and Elder, 2002).

2.1.4. Non-Bovine Sources

It is likely that reservoirs other than cattle contribute to the presence of *E. coli* O157:H7 subtypes in various geographical locations (Shere et al., 1998). Many non-bovine species have been identified as carriers of *E. coli* O157:H7 including horses, pigs, birds, deer, sheep, dogs, flies, pigeons, raccoons, rats, and insects (Chapman et al., 1997; Cizek et al., 1999; Hancock et al., 1998; Keene et al., 1997; Kudva et al., 1997; Kudva et al., 1996; Sargeant et al., 1999; Shere et al., 1998). Cattle frequently have contact with other animals in their environment and this contact, either directly or indirectly, may be a means of exposure to *E. coli* O157:H7 (Cizek et al., 2000; Rice et al., 2003; Sargeant et al., 1999; Van Donkersgoed et al., 2001) and continued maintenance of the pathogen in a herd.

2.1.4.1. Water

Water troughs could potentially be a non-bovine reservoir of *E. coli* O157:H7 on farms and in feedlots, with a prevalence ranging from 1.5 – 13 % (Faith et al., 1996; Hancock et al., 1998; Renter et al., 2003; Sargeant et al., 2000; Shere et al., 1998; Van Donkersgoed et al., 2001). Experimentally, *E. coli* O157:H7 persisted in 8 °C water for 91 D with only a 2 log decrease (CFU ml^{-1}) in population over this period (Wang and Doyle, 1998). *E. coli* O157:H7 has also been shown to replicate in water (Wang and Doyle, 1998) and it does appear that water sources play a role in the persistence of *E. coli*

O157:H7 infection within a herd. Similar *E. coli* O157:H7 isolates have been found in both water and cattle fecal samples on the same farm (Faith et al., 1996; Hancock et al., 1998). Fecal contamination of water likely leads to the perpetual re-infection of cattle, and the persistence of *E. coli* O157:H7 on the farm.

2.1.4.2. Feed

There is conflicting data regarding the prevalence of *E. coli* O157:H7 in cattle feed. *Escherichia coli* O157:H7 was not found in several surveys of cattle feeds (Hancock et al., 1998; Lynn et al., 1998), while other studies have found a proportion of feedstuffs to be contaminated (Bach et al., 2005; Buchko et al., 2000a; Dodd et al., 2003; Hancock et al., 2001; McAllister et al., 2006; Shere et al., 1998). Several studies have shown that *E. coli* O157:H7 can grow in silage and grain (Fenlon and Wilson, 2000; Lynn et al., 1998) with high lactate and low propionate concentrations being associated with enhanced growth in corn silage (Lynn et al., 1998).

Escherichia coli has been frequently isolated from cattle diets (30.1%; Lynn et al., 1998) but generic *E. coli* may not be a good indicator of the degree that diets can serve as an infectious source of *E. coli* O157:H7 (Dodd et al., 2003). A study by Dodd et al., (2003) showed no correlation between generic coliform counts and prevalence of *E. coli* O157:H7 in mixed feed samples. Adulteration of cattle feed with *E. coli* O157:H7 may play a limited role in the infection and reinfection of cattle. Unlike water, the prevalence of *E. coli* O157:H7 in feed does not appear to be associated with the seasonal prevalence of fecal shedding in cattle (Smith et al., 2001). This would suggest that the higher shedding levels found during the summer are not due to infection via contaminated

feedstuffs. Further, higher prevalence rates have been found in feeds that have been exposed to cattle (Dodd et al., 2003) than in feedstuffs that have not, suggesting that *E. coli* O157:H7 enters feed as a result of *contact* of the feed with cattle. Consequently, feeds that have not had direct contact with cattle are unlikely to be a source of *E. coli* O157:H7.

2.1.4.3. Manure and soil

Numerous studies have investigated the survival of *E. coli* O157:H7 in manure and soil (Avery et al., 2004; Bolton et al., 1999; Hutchison et al., 2005; Hutchison et al., 2004; Jones, 1999; Ogden et al., 2002). The spreading of farmyard manure (FYM) and fecal slurries from livestock production units onto agricultural land is a common waste management practice. However, this practice may result in contamination of food and water (Cieslak et al., 1993; Ogden et al., 2001; Pell, 1997) and contribute to the maintenance of this pathogen in a herd. Bolton et al., (1999) noted a 4 – 5 magnitude decrease in *E. coli* O157:H7 populations 50 D after the spreading of inoculated manure on grazing land. In another study, it took 63 D and 32 D for *E. coli* O157:H7 populations (approximately 10^6 CFU g^{-1}) to be undetectable in inoculated FYM from sheep and beef, respectively (Hutchison et al., 2005). Further, *E. coli* O157:H7 was detected for 8 weeks in soil core samples after application of inoculated waste to grassland (Avery et al., 2004).

2.1.5 Mitigation in Cattle

2.1.5.1. Farm Management

Cattle are grazing animals, and bacteria are ubiquitous in the environment. Elimination of *E. coli* O157:H7 from the environment is therefore unlikely but a variety of management practices have been investigated for their ability to minimize exposure of cattle to this pathogen.

2.1.5.1.1. Water Sources

Chlorination of water to reduce *E. coli* O157:H7 is successful *in vitro*, though it is not efficacious on-farm. Administration of chlorine at various concentrations decreased *E. coli* O157:H7 populations in water (LeJeune et al., 2001; Rice et al., 1999; Zhao et al., 2001). For example, the application of chlorine (5ppm) to an experimentally inoculated water source resulted in a 10^6 -10^7 decrease in *E. coli* O157:H7 (CFU/ml) (Doyle, 2003). Though *E. coli* O157:H7 has demonstrated minimal chlorine tolerance, the presence of organic material, in the form of rumen fluid or feces inactivates the bactericidal effect of chlorine. In the above mentioned study, the addition of rumen fluid (at a ratio of 100:1 water:rumen fluid) neutralized the ability of chlorine to kill *E. coli* O157:H7 (Doyle, 2003). Furthermore, on-farm surveys do not show an effect of chlorine on *E. coli* O157:H7 populations. The presence of organic material in water troughs may account for the lack of efficacy of chlorine in these studies. Samples collected from chlorinated and unchlorinated water troughs did not differ statistically in the frequency or numerical isolation of *E. coli* O157:H7 (LeJeune et al., 2004). As cattle frequently contaminate their water source with feces, chlorination may be an impractical method of controlling *E. coli* O157:H7 on farm

It is not clear whether water trough cleaning is beneficial for reducing *E. coli* O157:H7. Frequent cleaning would limit the build-up of organic material and reduce the level of *E. coli* O157:H7 present. Removal of organic material would also improve the efficacy of those mitigation strategies that are inhibited by ruminal and fecal contamination (Callaway et al., 2002). However, the frequency of trough cleanings required to minimize contamination may be unrealistic under production conditions, as water maybe re-contaminated by colonized cattle shortly after cleaning. *Escherichia coli* O157:H7 has also been shown to survive longer in sterile water than water from unsterile sources (Kerr et al., 1999; McGee et al., 2002). Various microbial populations existing in contaminated water may limit *E. coli* O157:H7 survival and replication by competitive exclusion or predation (Flint, 1987; Korhonen and Martikainen, 1991).

Sodium caprylate (120 nM) inactivated *E. coli* O157:H7 in water (Amalaradjou et al., 2006). Inclusion of cattle feces in the water slightly inhibited the effect of sodium caprylate, while the inclusion of feed amplified the effect. Palatability of the water as a result of inclusion of sodium caprylate was not tested.

Lactic and butyric acid have shown limited success in their ability to reduce *E. coli* O157:H7 in water (Doyle, 2003; Zhao et al., 2006). Various mixtures of lactic acid, acidic calcium sulfate, caprylic acid, butyric acid, and sodium benzoate, had little effect on *E. coli* O157:H7 levels in water containing rumen contents (100:1) *in vitro*, however greater concentrations of butyric acid ($\geq$2%) and caprylic acid ($\geq$0.1%) decreased *E. coli* O157:H7 levels by > 5 log CFU/ml in water (*in vitro*) after 20 minutes. These combinations were also bactericidal to *E. coli* O26:H11 and *E. coli* O111:NM in the presence of rumen fluid and cattle feces (Zhao et al., 2006). Unfortunately, administration

of these chemicals to water troughs significantly decreased the water intake of dairy heifers, making this an impractical mitigation strategy (Doyle, 2003; Zhao et al, 2006).

2.1.5.1.2. Feed

Thermal treatment of inoculated high and low energy feed rations resulted in decreased *E. coli* O157:H7 populations (Hutchison et al., 2007). The killing effect of the treatments increased with increasing temperature. Heating at 50 – 55 °C for 600 s resulted in reductions of approximately 1.6 logs. A 2.2 $\log_{10}$ decrease was found when rations were treated at 70 °C for 120 s. *Escherichia coli* O157:H7 survived heat treatment better in a high energy grain-based diet than a low energy diet at lower temperatures, however thermal destruction of *E. coli* O157:H7 at 70 °C was similar for both diets.

During storage of feed prior to treatment, decreases in *E. coli* O157:H7 populations were consistently noted in the low energy diet. This decrease in population was attributed to indigenous bacterial populations inhibiting *E. coli* O157:H7. The authors noted that naturally occurring *E. coli* O157:H7 in the feed might show greater resistance to treatment than inoculated strains due to their greater environmental fitness. This may extend to other environmental challenges as acid tolerant *E. coli* O157:H7 strains have also exhibited enhanced heat tolerance (Tosun and Gonul, 2005).

2.1.5.1.3. Housing conditions

Wet, muddy, and manure filled pens have been linked to the fecal shedding of *E. coli* O157:H7 by cattle (Smith et al., 2001; Stanford et al., 2005), likely due to the ability of *E. coli* O157:H7 to survive in manure (Kudva et al., 1998). In a survey of dairy

operations, dairy cattle housed in well drained pens were less likely to shed *E. coli* O157:H7 than those housed in pens in which manure and mud were allowed to accumulate (Stanford et al., 2005). Other practices such as flushing of alleys (Garber et al., 1999) and the development of dusty housing conditions (Zucker and Muller, 2000) may also promote the distribution of *E. coli* O157:H7 within housing environments.

2.1.5.1.4. Animal Density

Prevalence of *E. coli* O157:H7 culture-positive animals may also be positively correlated with animal density (Garber et al., 1995; Vidovic and Korber, 2006). Higher pen densities likely increase the spread of pathogens throughout a feedlot by increasing incidences of direct contact between shedding and non-shedding animals as well as the degree of environmental contamination (Vidovic and Korber, 2006). Other studies examining the prevalence of *E. coli* O157:H7 with respect to housing density in livestock did not identify a significant relationship (Hutchison et al., 2005; Rugbjerg et al., 2003). This could be due to differences in factors such as housing conditions, bedding types, and activity and age of the cattle. Reducing cattle density within feedlots may be one means of lowering the transmission and shedding of *E. coli* O157:H7.

2.1.5.1.5. Animal Sorting

Numerous studies have identified heifers as typically having the highest prevalence of *E. coli* O157:H7 among cattle groups (Garber et al., 1995; Hancock et al., 1994; Wilson et al., 1992). Stanford et al., (2005) found that calves and heifers had a higher prevalence of shedding of *E. coli* O157:H7 than adult dairy cattle. Alternately,

pens containing greater than 85% beef-type heifers have been associated with a reduced likelihood of the pen being positive for *E. coli* O157:H7 (Dargatz et al., 1997). In beef production, however, it is not feasible to separate cow-calf pairs, and as a result animal sorting cannot be employed as a management practice to lower transmission of *E. coli* O157:H7 within a herd.

2.1.5.2. Diet

Feedlot diets typically contain high levels of grain to optimize the efficiency of growth (Huntington, 1997). However, these starchy feedstuffs also alter the ruminal and intestinal environment. For this reason, numerous studies have looked at the effects of diet on the shedding and survival of *E. coli* O157:H7 (Bach et al., 2005; Berg et al., 2004; Buchko et al., 2000a; Garber et al., 1995; Hancock et al., 1997; Herriot et al., 1998; LeJeune et al, 2004; Russell and Rychlik, 2001; Sargeant et al., 2004).

2.1.5.2.1. Feedstuffs

Various feedstuffs have been shown to alter the fecal shedding of *E. coli* O157:H7. A number of studies have shown that feeding barley diets as compared to corn diets is associated with increased fecal shedding of *E. coli* O157:H7 (Berg et al., 2004; Buchko et al., 2000a; Dargatz et al., 1997; Herriott et al., 1998) whereas others have not identified such a relationship (Garber et al., 1995; Hancock et al., 1997). Buchko et al., (2000a) compared the *E. coli* O157:H7 status of yearling steers when fed one of three diets (85% cracked corn, 15% whole cottonseed + 70 % barley, or 85% barley). Levels of *E. coli* O157:H7-positive fecal samples were higher in cattle fed 85% barley than in cattle

fed the other two diets. Berg et al., (2004) found a higher prevalence of *E. coli* O157:H7-positive samples and a higher average magnitude of shedding (CFU/g) in cattle fed barley-based diets compared to cattle fed corn-based diets. Cottonseed meal, corn silage and beet pulp also have been shown to increase carriage (Sargeant et al., 2004) whereas whole cottonseed, clover and corn (Garber et al., 1995; LeJeune et al, 2004) have been shown to decrease shedding of *E. coli* O157:H7.

Acid resistance may be induced in *E. coli* by feeding high concentrate diets. Naturally, ruminants are grazers, and symbiotic microbes within the rumen accomplish digestion of fibrous plant material. Some microbes in the rumen are capable of fermenting sugars and starches with the majority of starch fermentation (microbial) occurring in the rumen (Russell and Rychlik, 2001). Grain may pass through the rumen undigested when animals are fed high concentrate diets, resulting in bacterial fermentation of starch in the colon and cecum. Consequently, colonic pH declines and starch-fermenting, acid-resistant bacteria predominate (Russell and Rychlik, 2001). *E. coli* O157:H7 is one such bacterium capable of starch fermentation and has demonstrated extreme acid resistance (Diez-Gonzalez and Russell 1997). Acidic conditions due to high volatile fatty acid concentrations, a characteristic of high-grain diets, did not affect survival of *E. coli* O157:H7 *in vitro* (Boukhors et al., 2002). However, Shin et al., (2002) showed that VFAs can inhibit the growth of *E. coli* O157:H7, though at VFA concentrations much higher than one would expect to find in any region of the bovine GIT (Ward et al., 1961).

Initial studies suggested that the fecal shedding of *E. coli* O157:H7 might be decreased when cattle are switched from a high concentrate to a roughage diet prior to

slaughter (Diez-Gonzalez et al, 1998). However, subsequent studies were unable to repeat this result (Callaway et al., 2003; Hovde et al., 1999; Kudva et al., 1999; Kudva et al., 1997) and exclusive feeding of hay diets has actually shown to prolong *E. coli* O157:H7 carriage (Hovde et al, 1999).

2.1.5.2.2. Fasting

As previously mentioned, starch digestion results in the production of VFAs, and these VFAs have been shown to have an inhibitory effect on *E. coli* O157:H7 (Shin et al., 2002). Cattle are often fasted prior to slaughter as a result of transport and lairage (Wood et al., 2006) and a number of studies have questioned if prolonged fasting leads to a higher prevalence of *E. coli* O157:H7 (Buchko et al., 2000b; Harmon et al., 1999; Jordan et al., 1999). Fasting has been shown to decrease VFA concentrations and increase pH in the rumen, but it did not have an effect on the ruminal or fecal populations of *E. coli* O157:H7 in calves (Harmon et al., 1999). Another study found that intermittent fasting after feeding a concentrate diet (80% barley) or a 100% forage ration (alfalfa) did not alter the shedding of *E. coli* O157:H7 in cattle (Buchko et al., 2000b). However, subsequent re-feeding of a 100% forage ration did significantly increase fecal shedding of *E. coli* O157:H7 (Buchko et al., 2000b). A simulation study by Jordan et al., (1999) did not predict prolonged fasting to have an impact on *E. coli* O157:H7 populations.

2.1.5.3. Probiotics

The term probiotic, as defined by Fuller (1989), encompasses commensal bacteria administered for the purpose of pathogen exclusion. Exclusion may be in the form of competition for attachment sites and nutrients, or antagonism by the production of anti-

microbial substances. The effectiveness of probiotic bacteria is enhanced when they multiply within the animal and integrate into the normal flora. They should also be able to survive extended periods of storage to facilitate use.

2.1.5.3.1. Colicins

Zhao et al., (1998) was the first to examine probiotics as a means of mitigating *E. coli* O157 in cattle. Previous work (Murinda et al., 1996) had demonstrated the existence of *E. coli* strains with anti-O157 activity as a result of the production of O157-antagonistic colicins. Colicins are antibiotics that are produced by, and target, *E. coli* strains in response to stress. Potential probiotic bacteria were isolated from cattle feces and GIT and tested for inhibitory activity against *E. coli* O157. By providing calves with a probiotic mixture (17 non-O157 *E. coli* isolates and one *Proteus mirabilis* isolate, isolated from cattle feces or cattle GIT tissue), Zhao et al. (1998) hypothesized that the probiotic bacteria would colonize the GIT and release anti-O157 compounds into the intestinal milieu. Probiotic treatment did in fact reduce the duration of ruminal carriage of *E. coli* O157:H7 in treated calves compared to the duration of carriage in control animals; however, the specific mechanism of inhibition was not addressed. Multiple probiotic treatments have been suggested as a means of improving the clearance of *E. coli* O157:H7 (Zhao et al., 1998).

The potential of colicin to inhibit *E. coli* O157:H7 improved with the isolation of colicins with anti-*E. coli* O157 activity (Jordi et al., 2001). This group also discussed the idea of "constructing" colicin-producing bacteria from resident ruminal non-*E. coli*

bacteria. Using non-*E. coli* bacterial strains would decrease the chance of conferring colicin production to *E. coli* O157:H7

Schamberger and Diez-Gonzalez (2002) investigated the presence of anti-O157 colicinogenic *E. coli* in species other than cattle. Based on the principle that the type of colicin produced is species-specific (Riley and Gordon, 1996), *E. coli* O157:H7 strains present in cattle should theoretically not be resistant to colicins produced by non-cattle *E. coli* isolates. The highest rate of *E. coli* isolates demonstrating activity against *E. coli* O157:H7 were collected from sheep and deer, with up to 63% of *E. coli* isolates inhibiting *E. coli* O157 (Schamberger and Diez-Gonzalez, 2002). This group also noted that inhibition was in fact partially due to the production of colicins, and not just competition for nutrients or binding sites. They demonstrated this by showing an increase in *E. coli* O157:H7 inhibition when *E. coli* O157:H7 was combined with colicinogenic bacteria versus when combined with non-colicinogenic bacteria. Further work showed that 10^8 CFU colicinogenic bacteria /g of feed could significantly reduce *E. coli* O157:H7 in cattle (Schamberger et al., 2004).

2.1.5.3.2. Lactic acid bacteria

Lactic acid bacteria can also decrease the fecal shedding of *E. coli* O157:H7. A number of *Lactobacillus* isolates, tolerant of acid (pH 2) and bile, significantly decreased *E. coli* O157:H7 in cattle manure and rumen fluid (Brashears et al, 2003a). Feeding a *Lactobacillus acidophilus* preparation decreased the fecal shedding and the contamination of hides with *E. coli* O157:H7 (Brashears et al, 2003b). Live weight gain and feed intakes did not differ with treatment. A subsequent study showed that two

Lactobacillus species inhibited *E. coli* O157:H7 attachment to intestinal epithelial cells by inducing intestinal cell expression of mucins (Mack et al., 1999). Mucins were shown to bind to *E. coli* O157:H7 inhibiting subsequent attachment to intestinal cells (Mack et al., 1999). Multiple studies have demonstrated the success of numerous lactobacilli capable of reducing *E. coli* O157:H7 carriage in cattle (Stephens et al., 2007; Younts-Dahl et al., 2005; Younts-Dahl et al., 2004). A recent study showed *L. acidophilus* alters the transcription of *E. coli* O157:H7 genes involved in colonization (Medellin-Pena et al., 2007).

A bifidobacteria was also shown to enhance specific and non-specific immune response in mice against *E. coli* O157:H7 (Shu et al., 2001). *Bifidobacterium lactis* has been shown to enhance phagocytic capacity of blood leukocytes and macrophage (Arunachalam et al., 2000; Gill et al., 2000) and increase intestinal IgA antibody response against various bacteria (Fukushima et al., 1999; Mestecky and Russell, 1998).

2.1.5.4. Tasco-14

Several studies have looked at the use of Tasco-14, an extract from the seaweed species *Ascophyllum nodosum*, in mitigating *E. coli* O157:H7 in cattle. Braden et al., 2004 determined that including 2% Tasco-14 in the diet of cattle significantly reduced the number of animals shedding *E. coli* O157:H7 in their feces. Tasco appears to directly affect the survival of *E. coli* O157:H7 in the animal GIT (Braden et al., 2004). More recently, Bach et al., (2008) reported that feeding steers *A. nodosum* (20g/kg diet DM) reduced the fecal shedding of *E. coli* O157:H7. A benefit to Tasco supplementation is the relatively short supplementation period (roughly 14 d) required to significantly reduce *E.*

coli O157:H7 shedding in comparison to the greater time requirement of other strategies such as the feeding of lactic acid bacteria.

2.1.5.5. Antibiotics

There is much debate over the use of antibiotics in cattle production, mostly due to concerns over the development of antibiotic resistance in numerous bacterial species. *E. coli* O157:H7 isolates have shown resistance towards cefazolin, cephalothin, osacillin, vancomycin, trimethoprim-sulfamethoxazole, ceftiofur, ampicillin, tetracycline, amoxicillin, florfenicol, spectinomycin, rifampin, tylosin (McAllister et al., 2006), penicillin, clindamycin, erythromycin, and tilmicosin (Galland et al., 2001). It is important to note, however, that most of these antibiotics are not targeted at *E. coli*.

The antibiotic neomycin sulfate has been shown to decrease fecal *E. coli* O157:H7 in cattle (Elder et al., 2002; Ransom et al., 2003). In fact administration of neomycin was shown to be more effective in reducing the fecal and hide prevalence of *E. coli* O157:H7 than vaccination or feeding of *L. acidophilus* (Woerner et al., 2007). Neomycin is commonly withdrawn from the diet for a period of 24 h prior to slaughter as a means of diminishing the presence of antibiotic residues in beef products. Though administration of this antibiotic has been effective, *E. coli* O157:H7 has shown resistance to neomycin (Stephan and Schumacher, 2001). Carriage of *E. coli* O157:H7 in cattle is asymptomatic and appears to have no negative health consequences for the animal. For this reason, and concerns regarding antibiotic resistance, it is highly unlikely that antibiotics will be used as a means of mitigating *E. coli* O157:H7 in cattle.

2.1.5.6. Immunization

Anti-*E. coli* O157 vaccines have been developed to target antigenic components of the pathogen's type III secretion apparatus and related proteins. Adherence to host cells is necessary for colonization. A pathogenicity island called the locus of enterocyte effacement (LEE) encodes proteins involved in the attachment of *eae*-positive *E. coli* O157:H7 to host cells. Vaccines targeting colonization factors encoded by LEE include anti-Tir, anti-EspA (type III *E. coli* secreted protein), and anti-intimin (Dziva et al., 2007; Potter et al., 2004).

A vaccine targeting both Tir and Esp was successful in reducing the number of culture positive animals as well as the duration of shedding as compared to control animals (Potter et al., 2004). A follow-up study tested the vaccine on a field scale. One hundred and nine feedlot pens of cattle from nine different feedlots were administered the vaccine. Contrary to the laboratory, results administration of this vaccine to cattle in feedlots did not change the proportion of cattle positive for *E. coli* O157:H7 (Van Donkersgoed et al., 2005). Discrepancies were attributed to variation in the manufacturers of the vaccine and the number of booster vaccinations administered. A second intimin-specific vaccine reduced the fecal shedding of *E. coli* O157:H7 in mice (Judge et al., 2004).

Subsequent attempts at vaccine-focused mitigation of *E. coli* O157:H7 have not been successful at reducing the fecal shedding of *E. coli* O157:H7 in cattle (Dziva et al., 2007; van Diemen et al., 2007). Though capable of inducing an immune response, vaccination did not protect cattle from colonization. The bacterial cell may not

continuously express adherence proteins and therefore immune attack may be limited to the time between the expression of such attachment proteins and host cell attachment.

The O157 antigen and various other antigens have also been the target of anti-*E. coli* O157:H7 vaccines. Most have been successful in inducing a specific immune response to the administered antigen, however vaccination against the O157 antigen has not reduced the shedding of *E. coli* O157:H7 by animals challenged with the bacteria (Conlan et al., 2000; Conlan et al., 1999). Vaccination still offers promise as a mitigation strategy for *E. coli* O157:H7 in cattle but further work is required to develop an efficacious feedlot vaccine.

2.1.5.7. Bacteriophage therapy and *E. coli* O157:H7

Bacteriophages are highly specific, bacterial viruses. Details regarding bacteriophages and their properties are found below (Section 2.2).

Bacteriophage specific to the O157 antigen of *E. coli* O157:H7 were isolated in 1999 by Kudva et al., and have been successfully isolated in many subsequent studies (Bach et al., 2003; Capparelli et al., 2006; Fischer et al., 2004; Mizoguchi et al., 2003; Morita et al., 2002; Muniesa and Jofre 2004; O'Flynn et al., 2004; Oot et al., 2007; Sheng et al., 2006; Tanji et al., 2004; Tanji et al., 2005).

Oral administration of O157-specific bacteriohpage quickly eliminated the pathogen from the gastrointestinal tract of mice (Tanji et al., 2005). *In vitro* work showed a slight decrease in the effectiveness of phage under anaerobic conditions versus aerobic conditions. However, *in vivo*, bacteriophage successfully eliminated *E. coli* O157:H7 in

murine hosts (Tanji et al., 2005). Subsequent studies were also able to eliminate *E. coli* O157:H7 from mice using bacteriophage specific to *E. coli* O157 (Sheng et al., 2006).

No effect of bacteriophage was found when lambs were orally administered 10^{13} PFU of bacteriophage DC22 two days post-inoculation with *E. coli* O157:H7 strain E318N (Bach et al., 2003). Bacteriophages were found in the feces of the treated sheep for eight days following administration. Bacteriophage DC22 required a high MOI (10^5 PFU/CFU) to efficiently lyse *E. coli* O157:H7, a property that is undesirable for a therapeutic phage. The authors also suggested that multiple doses of a cocktail of phage may have greater success in mitigating *E. coli* O157:H7.

The application of bacteriophage to challenged calves has produced conflicting results regarding effectiveness. Phage has been shown to completely eliminate *E. coli* O157:H7 in experimentally inoculated calves (Waddell et al., 2000). Treatment of calves with phage shortened the shedding of *E. coli* O157:H7 by six days as compared to calves that were inoculated with *E. coli* O157:H7 but not treated. In another study, however, the shedding of *E. coli* O157:H7 was only temporarily decreased with phage treatment (Goodridge and Chase, 2006). Both authors did note a sizeable increased in phage titre as *E. coli* O157:H7 counts declined. This would suggest that decreases in *E. coli* O157:H7 were influenced by the presence of virulent bacteriohpage. Dissimilarities in the effect of bacteriophage on *E. coli* O157:H7 populations in these two trials were likely due to differences in experimental design. A single application of phage was unable to significantly lower *E. coli* O157:H7 shedding (Goodridge and Chase, 2006) whereas multiple doses eliminated the organism from calves (Waddell et al., 2000).

Recently, administration of a two-phage mixture to the recto-anal junction of 5 six-month old steers decreased, but did not eliminate, the fecal shedding of *E. coli* O157:H7 (Sheng et al., 2006). The recto anal-junction had previously been identified as the primary site of *E. coli* O157:H7 colonization in cattle (Naylor et al., 2003). Rectal application of 10^6 PFU of the phage mixture to each steer, and a constant supply of phage in the drinking water, significantly reduced *E. coli* O157:H7 carriage (CFU/rectal swab) as compared to untreated steers. However, *E. coli* O157:H7 was still isolated from four of the five treated steers. Sheng et al., (2006) suggested that the lack of an immune reponse may be the reason why *E. coli* O157:H7 was not eliminated from the steers.

Phage-resistant bacteria have appeared during bacteriophage therapy (Smith and Huggins, 1982; Tanji et al., 2005), but careful selection of bacteriophage may minimize the development of resistance. Phage-resistant bacteria were noted in early *E. coli*/phage studies (Smith and Huggins, 1982). In this case, however, the bacteriophage utilized was specific to a capsular antigen involved in pathogenicity. This intuitive concept meant that if phage resistance developed, through loss or alteration of the target antigen, it would also be accompanied by a significant loss of virulence.

Bovine and ovine feces, as well as rumen samples, have been popular sources for the isolation of phage. The ovine rumen alone has been estimated to contain 3.9×10^9 and 1.4×10^{10} phage particles/ml of rumen fluid (Klieve and Swain, 1993). Isolation of phages from sources regionally distant from the intended recipient animals may be of particular advantage. Predominating *E. coli* O157:H7 strains are likely to differ among species and regions. Using phages from environments that differ from those of the host

may reduce the likelihood of targeted *E. coli* O157:H7 strains developing resistance, however, specificity of such phages to the target host may also be reduced.

Certain characteristics are required for phages to be useful in phage therapy. Phages must be specific to their target host cell. For example, when isolating phages specific to *E. coli* O157:H7, they are analyzed for their ability to lyse a culture of *E. coli* O157:H7 or to form plaques on a lawn of *E. coli* O157:H7 (Kudva et al., 1999). Of course, demonstrating that a phage possesses virulence towards *E. coli* O157:H7 does not dismiss the possibility that the phage may be virulent towards other serotypes. Therefore, it is necessary to test the ability of the phage to lyse other *E. coli* serotypes such as *E. coli* O111 and O5 (Kudva et al., 1999). Specificity is necessary when the intended targets of phages reside in the ruminant digestive tract, as inadvertent inhibition of bacteria involved in feed digestion would be an undesirable outcome of phage therapy.

Phages must also demonstrate a high virulence towards the target host cell for phage therapy to be efficacious. Collision between a virion and its target cell is necessary for infection and it is likely that such events occur infrequently within the large volume of digesta present in ruminants. When a collision does occur, it is necessary for adsorption and infection to take place. Virulence is often assessed by combining a known concentration of host cells with varying concentration of phage particles and determining the lowest PFU/ml at which complete lysis occurs (Johnson et al., 2006). The ratio of PFU/CFU is called the multiplicity of infection (MOI). Multiplicity of infection and the virulence of a bacteriophage are inversely related. An MOI of 10^3 PFU/CFU is generally considered to demonstrate a level of sufficient virulence for phage therapy to be successful (Kudva et al., 1999).

Phages demonstrating high levels of virulence are typically lytic, that is, they enter the host cell, take over host cell machinery, replicate and lyse the host cell upon release. Lysogenic, or temperate, phages can follow the lytic pathway, but more often, viral nucleic acid of the temperate phage is integrated into the genome of the host cell. Viral nucleic acid can remain in this state for many cycles of host reproduction, with no affect on the host. For this reason, lytic phages are much more effective for use in bacteriophage therapy than temperate phage.

Phages must survive delivery into the harsh conditions of the ruminant digestive tract, particularly, low pH (Tanji et al., 2005). *E. coli* O157:H7 has been shown to primarily colonize the lower intestinal tract of ruminants, and most recently, the recto-anal junction (Naylor et al., 2003). For infectious phage to pass to this site of colonization it must survive the microbially active environment of the rumen and the acidic environment of the abomasum. Some O157-specific phages are acid-tolerant with only a one-log decrease in titre at pH values as low as 3 (Johnson et al., 2006).

Finally, phages must not possess genes coding for virulence factors of pathogenic *E. coli* as it is possible for gene transfer to occur between the phage and the host (Brabban et al., 2005). The absence of such genes limits the risk of emergence of strains of *E. coli* O157:H7 with an even broader platform of virulence traits.

2.2 Bacteriophage

2.2.1. History

Discovery of the bacteriophage was shared by both Frederick William Twort and Felix Hubert d'Herelle. Twort was the first to document the phenomena of plaque

formation in 1915 (Twort, 1915), which he identified during an attempt to grow vaccinia virus. Twort noticed that contaminating colonies of micrococcus appeared to be diseased, exhibiting "watery-looking" areas on agar media (Twort, 1949). Microscopic investigation of the afflicted bacteria showed that the cells had degenerated into granules. He also determined that the disease-causing agent was filterable and therefore very small and he speculated that it was a fluid form of life or "an enzyme with the power of growth" (Twort, 1915).

Between 1917 and 1915 d'Herelle made similar observations, but came up with the hypothesis that a virus, capable of infecting bacteria, was the cause of such occurrences (d'Herelle, 1949; Institut Pasteur, 2007). During both a study of diseased locusts and the investigation of a dysentery epidemic, d'Herelle observed bactericidal occurrences such as transparent regions on bacterial lawns as well as clearing of once turbid cultures when combined with the filtrate of samples containing the isolated pathogen. At this point d'Herelle hypothesized that the bactericidal effect was due to a filterable virus that was parasitic to bacteria (d'Herelle, 1949). There was significant debate regarding d'Herelle's hypothesis of a bacterial virus until it was confirmed after the advent of the electron microscope (d'Herelle, 1949).

2.2.2. Structure

The virion (the mature, extracellular virus) is typically composed of nucleic acid, known as the core, and a protein coat, known as the capsid. This complex, composed of the capsid and core, is also known as the nucleocapsid. In some cases, the nucleocapsid is

further enclosed in an envelope or it may even have appendages (Bradley, 1967) as is the case for T4 bacteriophage.

2.2.2.1. Core

The nucleic acid of a virus, called the core (Bradley, 1967), may be composed of single-stranded (ss) or double stranded (ds) deoxyriobonucleic acid (DNA), or even ss or ds ribonucleic acid (RNA) (Ackermann, 2006). The core of bacteriophage T4 is composed of ds linear DNA, 169,903 bp in length (Miller et al., 2003). Instead of cytosine, T4 DNA contains hydroxymethylcytosine (HMC) residues, which are often glycosylated (Miller et al., 2003). Both of these features help to prevent restriction of viral DNA by host and viral restriction enzymes. Glycosylation and HMC deficient mutants also show altered regulation of transcript termination (Drivdahl and Kutter, 1990), DNA replication (Yee and Marsh, 1985) and packaging (Youil et al., 1995). Each T4 phage particle contains approximately 103% of the entire phage genome, resulting in terminal redundancy at each end of the molecule (Streisinger, 1966). Though the T4 genome is linear, circular permutations arise making the genome appear circular.

2.2.2.2. Capsid

The viral capsid encasing nucleic acid is composed of multiple protein subunits arranged in a regular fashion. In 1956, Crick and Watson hypothesized that due to the small size of known viral genomes, the protein shell must be composed of multiple small protein units, most likely the product of one gene, as opposed to one or two larger proteins. Electron microscopy did in fact demonstrate that the viral capsid is composed

of multiple protein subunits. These protein clusters, called *capsomers*, are themselves composed of identical protein sub-units called *structure units* (Lwoff et al., 1962).

Viral capsomers are arranged symmetrically, another observation that supported the hypothesis of Crick and Watson (1956). The entire virion can be categorized as cubic, helical, binary (cubic head with helical tail), or pleomorphic (Ackermann, 2006). Virion symmetry is a major factor considered in the classification of bacteriophages, along with nucleic acid type (Bradley et al., 1967; Lwoff et al., 1962). The wild-type symmetry of bacteriophage T4 is binary, with an elongated icosahedral head of triangulation class $T = 13$ (Aebi et al., 1974). The T4 capsid is composed primarily of protein gp23 along with at least 9 other polypeptides (Mosig and Eiserling, 2006).

2.2.2.3. Tail and tail fibers

In addition to the icosahedral head, the T4 phage particle also possesses a helical tail, made up of the tail tube (inner and outer tube), baseplate wedge and baseplate hub, as well as long and short tail fibers resulting in a "spider-like" appearance. Twenty-six proteins are required for construction of the tail and tail fibers (Mosig and Eiserling, 2006). The tail fibers are involved in adsorption of the free virus particle to the host cell while the baseplate-tail complex delivers the viral nucleic acid to the host cell membrane.

2.2.3. Classification

A method developed for the classification of viruses, proposed by Bradley (1967) is still widely used for the classification of phages. Symmetry and type of nucleic acid identifies the order or family of which a phage belongs. T4 belongs to the order

Caudovirales (currently the only order of bacteriophages recognized by the International Committee on Taxonomy of Viruses), which is composed of phages with cubic heads, helical tails, and ds linear DNA (Ackermann, 2006; Lwoff et al., 1962). Subclassifications for T4 and other *E. coli*-specific phage places them in the family of *Myoviridae*, and into the genus of "T4-like" viruses (Ackermann, 2006).

2.2.4. Viral replication of T4-like bacteriophages

There are five major steps involved in viral replication: 1) adsorption; 2) release of nucleic acid; 3) expression and replication of nucleic acid; 4) assembly, and 5) release. Viral replication is unlike replication in prokaryote and eukaryote cells, as viruses cannot replicate without a host. A great deal of research has been done on bacteriophage T4, an *E. coli* virus. For this reason, and because of its relevance to the research in this thesis, this summary of viral replication will focus on lytic T4-like bacteriophages.

2.2.4.1. Adsorption

Adsorption is a highly specific process but initial interaction between the virus particle and the host is random and reversible. Collision between the viral particle and susceptible host often results in an initial electrostatic attachment (Duckworth, 1987). Bacteriophage T4 tail fibers are released from their stored position and extend into a conformation conducive to association with lipopolysaccharides (LPS) or proteins on the host cell surface (Goldberg et al., 1994). Movement along the cell surface occurs until at least three long tail fibers bind, which causes a conformational change to the viral baseplate. Short tail fibers are then released and bind irreversibly to surface LPS (Mosig and

Eiserling, 2006). Although T4 phage bind specifically to LPS, other phages may be specific to surface proteins such as OmpC, as is the case for *E. coli*-specific phage SP21 (Tanji et al., 2004). Often divalent cations such as Mg^{2+} and Ca^{2+} are required as cofactors for adsorption (Puck et al., 1951; Guttman et al., 2005). Modification or absence of cell-surface receptors results in bacterial resistance to the virus. In such cases the phage cannot bind specifically to the host cell. *E. coli* strains expressing truncated O157 antigens and O157-deficient mutant strains were both resistant to O157-specific phages KH1, KH4, and KH5 (Kudva et al., 1999). Of course mutations in bacterial cells that result in increased resistance to bacteriophages are sometimes countered by mutant phages that alter their method of association with the resistant host (Parker, 2000).

The rate of phage adsorption is a function of host density. As host densities increase, so does the rate of phage adsorption (Abedon et al., 2001), while the average time of phage adsorption decreases. This is likely due to increased incidences of random collision among viral particles and the host when host densities are high. Higher adsorption rates are also associated with shorter latent periods (the time between phage adsorption and host cell lysis), as the optimal latent period (min) decreases with increasing host density (Abedon et al., 2001). However, the magnitude of this change in latent period with varying host density remains to be defined (Abedon et al., 2001, Wang et al., 1996).

2.2.4.2. Release of nucleic acid

Bacteriophages differ from animal viruses in the separation of nucleic acid from the protein coat. Often with animal viruses, the entire virus (including the capsid) is taken

up into the animal cell prior to release of the nucleic acid from the capsid (Luria et al., 1978). This is not the case for T4-like bacteriophages, which commonly deposit their nucleic acid at the cell membrane for transport into the cytoplasm (Mosig and Eiserling, 2006).

Following irreversible attachment of the T4 virus particle to the host cell, it has been proposed that a needle-like structure, centered under the viral baseplate penetrates the cell envelope (Kanamaru et al., 2002). A protein (Gp5) within the needle contains a lysozyme domain that likely digests bacterial peptidoglycan (Mosig et al., 1989). This action enables the tail tube to penetrate the cell wall, which coincides with the contraction of the tail sheath (Kellenberger, 1980; Moody and Makowski, 1981). A conformational change in the phage allows release of the DNA. Though much of the mechanism is unknown, DNA transfer through the cell membrane likely involves a transmembrane channel (Goldberg et al., 1994), proton-motive force (Goldberg et al., 1994) and possibly the help of membrane-altering proteins (Zoon et al., 1976).

2.2.4.3. Expression and replication of nucleic acid

Transcription of viral nucleic acid within the host cell can be broken up into three stages, commonly called early (immediate early), middle (delayed early) and late transcription.

Early genes are transcribed by host RNA polymerase (in the case of DNA viruses such as T4) as a result of the enzyme's recognition of very strong viral promoters. At this time RNA polymerase uses unmodified δ^{70}, but the polymerase core may be altered by a viral protein (Ross et al., 1993) that is delivered along with the nucleic acid. As early

proteins are produced, a cascade of RNA polymerase modifications occur, further promoting viral transcription and preventing host gene transcription. Additional phage gene products are involved in protection of the phage genome and proteins from destruction by nucleases, proteases, and other host defense mechanisms (Guttman et al., 2005).

Middle transcription results in the expression of proteins involved in viral nucleic acid replication including enzymes of the deoxyribonucleotide biosynthesis complex. These include proteins involved in recombination, protection of viral nucleic acid as well as RNA polymerase modifiers, RNases, and primers for late transcription (Mosig and Eiserling, 2006).

Like transcription, T4 genome replication is also temporal. Although multiple regions of replication exist, the initiation of replication is associated with a single origin (Dannenberg and Mosig, 1983). Replication is carried out in the direction of 5' to 3' (leading and lagging), by a viral-encoded replisome. As mentioned previously, the viral core contains approximately 103% of the phage genome. Intramolecular, and later intermolecular, recombination is utilized to solve the problem of replicating the 5' end of the lagging nucleic acid strand, a problem characteristic of 5' to 3' replication (Mosig, 1998; Mosig et al., 2001). Late replication therefore relies considerably on recombination, resulting in the formation of circular and branched intermediates that give rise to concatemeric strands of the viral genome.

Late genes code for products involved in the synthesis and release of new phage particles. These include proteins involved in DNA recombination and repair, proteins

involved in the assembly of the capsid, tail and tail fiber (morphogenesis proteins), as well as enzymes that lyse the host cell wall (Mosig and Eiserling, 2006).

2.2.4.4. Assembly

Phage assembly begins only after head, tail and tail fiber proteins are all synthesized by individual pathways. Assembly is initiated as a result of protein interactions (Duckworth, 1987). In fact, in the presence of additional gene products (Wood and Edgar, 1967), isolated protein components, and nucleic acid have been shown to assemble *in vitro* to form virulent particles (Edgar and Wood, 1966).

Viral DNA is bound by a terminase protein, which cuts the appropriate length of nucleic acid to be included in the phage head from the DNA concatemer (Rao et al., 1992). This protein-nucleic acid complex then binds to a portal protein present in the phage head forming a complex known as a "packasome." Adenosine triphosphate is hydrolyzed during the loading of the nucleic acid into the viral head (Black and Showe, 1983). Since approximately 103% of the genome is cut from the DNA concatemer and loaded into the phage head, each resultant phage particle will receive a different sequence of redundant genetic material at either end of the molecule (Thomas and MacHattie, 1967).

2.2.4.5. Release

Lysis of the bacterial cell is required for the release of newly synthesized phage. In T4 infection, T4 lysozyme, cleaves the bond between the *N*-acetylglucosamine and the muramic acid in the murein of the bacterial cell wall (Matthews, 1983). This lysozyme is

closely related to the lysozyme of the viral baseplate that digests the host cell wall in order to deliver the viral genome to the cell membrane (Kao and McClain, 1980; Paddison et al., 1998).

2.3 Conclusions

The need to control *Escherichia coli* O157:H7 populations in cattle is evident as the human health risk associated with the harboring of this pathogen by ruminant animals is high. However, ruminant digestion is dependent on a large resident microbial population and therefore any anti-microbial strategy used to control *E. coli* O157:H7 must be highly specific so as to not alter natural microfloral. Many of the mitigation strategies discussed lack specificity in their actions. However, bacteriophage therapy is highly specific due to the nature of adsorption to the targeted host. Also, bacteriophages occur naturally within the ruminant GIT and are easily isolated. For these reasons, bacteriophage therapy may offer an efficacious means for controlling *E. coli* O157:H7 in cattle.

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Chapter 3

3. BACTERIOPHAGE THERAPY FOR CONTROLLING *ESCHERICHIA COLI* O157:H7 IN FEEDLOT CATTLE: EFFECT OF ORAL VS. RECTAL ADMINISTRATION[1]

3.1. INTRODUCTION

Escherichia coli O157:H7 is a human pathogen with symptoms of infection ranging from watery diarrhea to hemorrhagic colitis and hemolytic uremic syndrome (PHAC, 2007). In some cases infection has resulted in death (Griffin and Tauxe, 1991). Sources of *E. coli* O157:H7 include, but are not limited to, undercooked ground beef, vegetables, and drinking water (Erickson and Doyle, 2007). Ruminant animals, particularly cattle, have been identified as a primary reservoir of *E. coli* O157:H7 (Borczyk et al., 1987; Hancock et al. 1994; Orskov et al., 1987; Rasmussen et al., 1993). Carriage of *E. coli* O157:H7 in cattle is asymptomatic (Cray and Moon, 2005) making it impossible to identify carrier animals without employing laboratory methods. Carriage is also transient and often seasonal (Rahn et al., 1997; Van Donkersgoed et al. 2001). Contamination of hides and shedding of *E. coli* O157:H7 have also been correlated with adulteration of meat and in particular with ground beef products (Elder et al. 2000). Control of the organism prior to slaughter is therefore necessary to reduce food contamination and subsequent human illness. A recent study indicated that 9% of *E. coli* O157:H7-positive animals in the feedlot shed *E. coli* O157 at levels greater than 10^4

[1] A version of this paper has been submitted for publication in the Journal of Food Protection.

CFU/g feces and that these so called "super-shedders" (Omisakin et al. 2003) accounted for 96% of all *E. coli* O157:H7 shed at the abattoir.

Bacteriophages are highly specific viruses that target bacteria. Renewed interest in bacteriophage therapy has led to its application for controlling various bacterial infections. As efficacy of phage therapy is related to the density of the host, this approach may be particularly suited for controlling *E. coli* O157:H7 in super-shedders. Kudva et al, (1999), were the first to isolate phages specific to the O-antigen of *E. coli* O157:H7 for future phage therapy use. Bacteriophage specific to *E. coli* O157 have since been administered to a variety of animals, including mice, calves, and sheep, with varying degrees of success in mitigating *E. coli* O157:H7 (Bach et al. 2003; Sheng et al. 2006; Tanji et al. 2005; Waddell et al. 2000).The recto-anal junction was identified as the primary site of *E. coli* O157:H7 colonization in cattle (Naylor et al. 2003). Two studies have since used various mitigation strategies to target this area (Naylor et al. 2007; Sheng et al. 2006). Application of bacteriophages directly to the recto-anal junction and concurrently in drinking water was successful in reducing, but not eliminating, the fecal shedding of *E. coli* O157:H7 by cattle experimentally infected by inoculation at the recto-anal junction (Sheng et al. 2006).

The objective of this study was to compare oral and/or rectal administration of a four strain O157-specific bacteriophage cocktail to feedlot steers with respect to establishment of phage populations within the animals and mitigation of experimental oral *E. coli* O157:H7 infection. Persistence and transmission of phage and *E. coli* O157:H7 within the feedlot environment were also monitored.

3.2. MATERIALS AND METHODS

3.2.1. Experimental animals and design.

Thirty-two Hereford X Angus steers (386 ± 29 kg average weight) were randomly assigned to four groups of eight in eight feedlot pens (two pens of four animals per treatment group) with replicate pens sharing a water source. The steers were cared for in accordance with Canadian Council on Animal Care guidelines (CCAC, 1993). They were acclimated to the new environment and a finishing diet consisting of 20% barley silage, 75% barley grain and 5% supplement (DM basis) for two weeks prior the start of the trial. Fecal and environmental samples were obtained during the acclimation period to confirm the absence of nalidixic acid-resistant (Nal^R) *E. coli* O157:H7 strains as well as *E. coli* O157:H7-specific bacteriophages. On day (D) 0, all cattle were inoculated via stomach tube with a five-strain mixture of Nal^R *E. coli* O157:H7, as described below. On Days -2, 0, 2, 6 and 9, steers were treated orally (ORL group), rectally (REC group), or both orally and rectally (O+R group) with a cocktail of four lytic *E. coli* O157:H7 bacteriophages, as described below. Cattle inoculated with *E. coli* O157:H7 but not treated with bacteriophages served as the control (CON) group and were sham-treated orally with phosphate buffered saline (PBS) and rectally with a 5 % methocel gel preparation.

3.2.2. Bacterial inoculum.

Five strains of Nal^R *E. coli* O157:H7, E318N (human origin), R508N (bovine origin), CO281-31N (human origin), E32511 (human origin), and H4420N (bovine origin), were combined for the bacterial inoculum. Each strain was cultured separately in

10 ml of tryptic soy broth (TSB, Difco, Ottawa, ON) containing 50 μg/ml nalidixic acid (Sigma Chemical Co., Oakville, ON, Canada) for 6 hours at 37°C with shaking at 150 rpm, then 100 μl was subcultured separately in 100 ml of TSB for 18 h at 37°C and 150 rpm. Each strain was enumerated by direct plating onto sorbitol MacConkey agar supplemented with 0.05 mg/L cefixime, 2.5 mg/L potassium tellurite and 50 μg/ml nalidixic acid (CT-SMACnal Dalynn Biologicals, Calgary, AB, Canada) for 18 h at 37°C. Plates with between 30 and 300 colonies were used for the determination of bacterial populations. Strains were then combined in equal concentrations and 52.3 ml of the mixture, totaling 5 x 10^{10} CFU *E. coli* O157:H7 (enumerated as above), was drawn into a 60 cc syringe for oral administration to each animal via stomach tube. Inoculations on D 0 were followed by the delivery of three 50 ml aliquots of PBS to rinse the stomach tube.

3.2.3. Bacteriophage preparation and administration.

Bacteriophages rV5, wV7, wV8 and wV11 (Public Health Agency of Canada, Laboratory for Foodborne Zoonoses, Guelph, Ontario, Canada), virulent for *E. coli* O157:H7, were propagated for 16 h at 37°C in *E. coli* O157:H7 R508, which was grown in TSB containing 2 mM $MgSO_4$ (Sigma, Oakville, ON). The resulting cultures were centrifuged for 10 min at 12,000 × *g*, and the supernatants were passed through 0.2 μm filters and titrated by plaque assays (Sambrook,1989). These preparations, containing approx 10^{10} PFU/ml, were combined to create the administered bacteriophage mixtures. For oral administration, the bacteriophage mixture containing a total of 3.3x10^{11} PFU in 35.2 ml PBS, was given via stomach tube, preceded by 30% (w/v) magnesium oxide in

100 ml of water and three 100 ml rinses of distilled water, and followed by 100 ml of 7.5% sodium bicarbonate buffer. Rectal treatment followed digital evacuation of feces from the rectum. The bacteriophage mixture ($1.5x10^{11}$ PFU) was suspended in 52 ml of 5% (w/v) methocel gel (Wiler PCCA, London, ON, Canada), which was applied directly onto to the recto-anal junction mucosa using a modified 60 cc syringe with part of the narrow tip removed to create larger opening. The O+R steers, treated both orally and rectally, received a total of $4.8x10^{11}$ PFU bacteriophages on each treatment day. Steers in the CON group were treated by oral dosage with PBS instead of bacteriophages. All steers received the planned oral, rectal, oral+rectal or sham oral treatment each treatment day.

3.2.4. Detection and enumeration of *E. coli* O157:H7.

Fecal samples were collected weekly during the two-week adaptation period, and on D 0, 1, 3, 5, 7, 9, 13, then once per week for the remainder of the 12-week experimental period. Samples were collected from each animal by digital rectal retrieval, changing gloves between each animal. Samples were transported in sterile, 100 ml polypropylene containers and were processed within 1 h following collection. Fecal samples collected during the adaptation period were assessed for the presence of Nal^R and naturally occurring strains of *E. coli* O157:H7 as follows. Enrichment of 10 g feces in 9 volumes of modified TSB (mTSB, BDH, Toronto, ON, Canada) containing 20 mg/L novobiocin (mTSB+n) for 6 h at 37°C was followed by immunomagnetic separation (IMS) using Dynabeads anti-*E. coli* O157 (Invitrogen, Burlington, ON, Canada). Following IMS, a 50-µl aliquot of the antibody-coated bead suspension was plated onto

CT-SMACnal and incubated for 18 h at 37°C. Any suspect *E. coli* O157:H7 colonies were tested for the O157 antigen by latex agglutination (Oxoid *E. coli* O157 Latex Kit, Oxoid, Nepean, ON, Canada). Agglutination-positive, putative *E. coli* O157:H7 colonies were plated for single colonies on Blood Agar (5% sheep blood, Dalynn Biologicals, Calgary, AB, Canada) and stored at 4°C.

For direct detection and enumeration of *E. coli* O157:H7 in feces post-inoculation, 1 g of feces was serially diluted ten-fold in PBS, thoroughly mixed by vortexing, and 100 µl volumes were spread-plated in duplicate onto CT-SMACnal plates and incubated for 18 h at 37°C. Whenever possible, plates containing 30 to 300 Nal^R colonies with typical morphology were used for plate counts and the average of the counts of duplicate plates were used to determine the levels of *E. coli* O157:H7 in the original sample (CFU/g). When sufficient colonies were not found after direct plating of 100 µl of the 1:10 fecal/PBS slurry, 1 ml of the same slurry was plated onto CT-SMACnal in a larger, 150mm x 15mm Kirby plate (Dalynn Biologicals, Calgary, AB, Canada), and processed as were the smaller plates. Once *E. coli* O157:H7 was no longer detectable by direct plating, 10 g of feces was enriched and tested by IMS as described above, with plating of the beads onto CT-SMACnal. Three agglutination-positive, putative *E. coli* O157:H7 colonies from each sample were then isolated by streaking onto Blood Agar (5 % sheep blood, Dalynn Biologicals, Calgary, AB, Canada) and stored at 4°C.

3.2.5. Detection and enumeration of bacteriophages.

Fecal samples (1 g) were mixed thoroughly in 9 ml of PBS and 1.8 ml aliquots were centrifuged at 10,000 X g for 10 min to sediment fecal material. The supernatant was filtered (0.8/0.2 µm Supor® membrane syringe filter, Pall Corporation, Mississauga, ON, Canada) to remove bacterial cells. For the detection of *E. coli* O157:H7 bacteriophages potentially in low numbers, as in samples collected before treatment or later in the study, 450 µl of the phage filtrate was incubated for 1 h at 37°C with 50 µl *E. coli* O157:H7 (R508N), to allow replication of any lytic bacteriophages. Four droplets (20 µl each) of the phage/*E. coli* O157:H7 mixture were then spotted on Modified Nutrient agar (Sambrook, 1989) Plates were incubated for 18 h at 37°C and examined for plaques.

Bacteriophages were enumerated by soft agar overlay plaque assays (Sambrook, 1989). Briefly, phage filtrate (40 µl) was diluted in 360 µl lambda diluent (Sigma, St. Louis, MO, USA) followed by serial dilution (1:10) as necessary. Diluted filtrate (100 µl) was then added to 100 µl *E. coli* O157:H7 (R508) and incubated for 20 min at 37°C to allow bacteriophage to attach. After incubation, 3.5 ml molten 0.7% UltraPure agarose (Invitrogen, Burlington, ON, Canada) was added to each sample. Tubes were mixed through inversion then poured onto duplicate pre-hardened MNA plates and incubated at 37°C for 12-18 h. Plates with plaque counts between 10 and 500 were used to determine PFU/ml.

3.2.6. Environmental samples.

To determine the presence and persistence of *E. coli* O157:H7 and bacteriophages in the environment, samples of feed, water and manure were collected weekly throughout the twelve-week experimental period. Samples were also obtained during the adaptation period and assessed for the presence of Nal^R *E. coli* O157:H7 and *E. coli* O157:H7 bacteriophages. All samples were collected 5-6 hours after morning feed delivery and were analyzed 1 h after collection. Five 20 g feed samples were taken from different areas of each feed bunk and combined. A portion (10 g) of the pooled sample was measured into 90 ml mTSB with novobiocin (mTSB+n) and stomached for 30 sec at 230 rpm. The samples were then incubated for 6 h at 37°C prior to detection of *E. coli* O157:H7 by IMS (as above). A second 10 g portion of the pooled feed sample was measured into 40 ml lambda diluent and stomached for 30 sec at 230 rpm, 1.8 ml was removed and used for detection of bacteriophage as described above. Water samples (120 ml) were collected weekly from each of the four water troughs after thorough mixing. For detection of *E. coli* O157:H7, a portion of each sample (100 ml) was filtered through a 100 ml micro-funnel (0.2 µm filter units; Pall Life Sciences, VWR, Mississauga, ON, Canada). The filter was then removed and placed into 100 ml mTSB+n and stomached for 30 sec at 230 rpm (Stomacher 400 Circulator, Seward), and processed for detection of *E. coli* O157:H7 as for feed samples. For detection of *E. coli* O157:H7 bacteriophages, 1.8 ml of each stomached filter sample was tested as described above for fecal filtrates. Manure samples (10 g) from three random fecal pats within a pen were collected weekly and pooled. Sub-samples (1 g) were tested for *E. coli* O157:H7 and bacteriophages as described for fecal samples, above.

3.2.7. Pulsed field gel electrophoresis of isolates.

Fecal Nal^R *E. coli* O157:H7 isolates from D1 (n = 60), and D 69, 76, and 83 (n = 58), as well as fecal pat (n = 14), feed (n = 2) and water (n = 1) isolates were grown on Tryptic Soy Agar (TSA, Difco, Ottawa, ON, Canada). Plates were incubated for 18 h at 37°C and isolates were prepared for PFGE as previously described by Bach et al., (2003). Analysis of *Xba*I-digested genomic DNA was performed according to the One-Day Standardized Laboratory Protocol for Molecular Subtyping of *Escherichia coli* O157:H7 (CDC, 1996). Resulting banding patterns were analyzed and compared using Bionumerics software to determine the genetic relatedness of the isolates following methods previously outlined by Tenover et al., (1995).

3.2.8. Microplate Phage Virulence Assay.

To determine if Nal^R *E. coli* O157:H7 isolates that differed from the inoculated strains in PFGE profiles were resistant to therapeutic bacteriophages, each of the five inoculated *E. coli* O157:H7 strains and four fecal isolates with PFGE profiles distinct from the inoculated strains were analyzed for their susceptibility to the four administered phages by a phage virulence assay in 96-well microplates. In this assay, the virulence of a phage for a given bacterial strain is assessed as the lowest multiplicity of infection (ratio of phage to bacteria, MOI) that results in complete lysis of a fixed number of bacteria 5 h after infection. Highly susceptible bacteria are completely lysed at low MOIs, while resistant bacteria may be lysed only partially or not at all, even at very high MOIs. Briefly, titres of the four bacteriophage stocks and the five bacterial strains,

prepared as described above, were determined by plaque assays and standard plate counts, respectively. Twenty microlitre volumes of each phage were serially diluted 10-fold in 180 µl volumes of TSB containing 10 mM $MgSO_4$ in wells of columns of 96-well microplates. Duplicate wells of each phage dilution were then inoculated with 20 µl the test bacterial culture, and the plates were incubated at 37°C for 5 h. Controls in each microplate included wells containing the TSB diluent inoculated with bacteria only, as a positive control for bacterial growth, with phage only, as a negative control, and with phage and a known susceptible bacterial strain, as a positive control for complete lysis. After incubation, the wells were visually inspected for turbidity due to bacterial growth and the highest dilution of phages to show complete lysis (no discernable turbidity) of each strain was recorded. The MOI at this dilution was calculated from the known titres of each phage and bacterial strain and normalized to the MOI required for complete lysis of 10^6 CFU of each strain. Strains requiring MOIs greater than 100-fold higher than the MOI of the same phage causing complete lysis of the inoculated strains were scored as resistant.

3.2.8. Transmission electron microscopy. Filter purified phage wV7 (1.0 ml, $1.38x10^8$), representative of the four administered phages all of the family *Myoviridae*, was centrifuged at 21.1 g (14.8K rpm) for 40min. The supernatant was decanted, and the pellet was washed by pipetting 200 µl sterile lambda diluent down the side of the tube, and immediately decanting the diluent off. Lambda diluent (800 µl) was then added to the phage pellet and the sample was mixed via vortexing. One droplet of phage solution was used for transmission electron microscopy (TEM).

For isolation of newly isolated phages forming large plaques with concentric bull's eye rings, plaque stabs, using 20µl sterile pipette tips, were taken from the centre of the lytic plaques on a dilution master plate and transferred to 1.5 ml sterile microcentrifuge tubes with sterile lambda diluent (1 ml). Samples were incubated at room temperature with gentle shaking (180 rpm) for 2 hours and then allowed to stand at 4°C overnight. Three subsequent serial dilutions, 10^0, 10^{-1} and 10^{-2}, (lambda diluent, 1 ml aliquots) were made in 1.5 ml centrifuge tubes. Dilutions were plated using the traditional overlay method described above and plates were incubated at 37 °C overnight. Sterile ultrapure H_20 (20µl) was pipetted directly onto a single plaque. A prepared TEM grid was then placed on top of the droplet for 1 min before being carefully removed and stained.

Formvar coated, copper mesh TEM grids (Pelco IGC 300) were placed on the sample droplets for 1 min. Excess moisture was removed by touching the edge of the grid to a piece of filter paper at a 45° angle. One drop of 2% uranyl acetate was placed on the grid and quickly removed with filter paper as described above. Samples were air-dried for 5 min prior to viewing with TEM (JEOL 100CX) at an image magnification of 50K. Resulting images were captured on film (Kodak 4489, 3.25" x.4" EM).

3.2.9. Statistical Methods.

For statistical analysis of enumeration data, all direct counts (CFU or PFU/g) were transformed to $\log_{10}$ values. Samples positive for *E. coli* O157:H7 or bacteriophages only by enrichment were assigned random values between 0 and 1 $\log_{10}$ CFU/g or PFU/g. For frequency and prevalence data, samples positive for *E. coli*

O157:H7 or bacteriophages by either direct or enrichment tests were scored as positive. Individual shedding levels (CFU or PFU/g) over the course of the trial and within a treatment group were averaged to determine the mean treatment shedding level for each treatment group. Fecal prevalence of phage and *E. coli* O157 was determined by calculating the percent of positive samples out of the total number of samples collected from individual animals within a treatment group. Descriptive statistics were generated using the FREQ procedure of SAS (Statistical Analysis System, 1999, SAS Institute, Cary, NC). The least squares means (LS-Means) function of the MIXED model procedure of SAS, using repeated measures, was used to compare the individual and mean treatment shedding levels of Nal^R *E. coli* O157:H7 and bacteriophage from fecal grab and environmental samples. Odds ratios were also calculated for the prevalence of Nal^R *E. coli* O157:H7 and bacteriophage-positive samples using the GLIMMIX procedure of SAS with CON cohorts as the referent. Mean treatment shedding levels were compared using the LSMEANS function within the repeated measures MIXED model procedure of SAS. Statistical significance was determined at $P < 0.10$.

3.3. RESULTS

Prior to the beginning of the trial, all fecal and environmental samples were negative for Nal^R *E. coli* O157:H7, *E. coli* O157:H7 and *E. coli* O157-lytic bacteriophages. After administration of the phages, all treated animals shed bacteriophages in their feces. Mean fecal phage levels were greatest in the O+R (7.38 log_{10} PFU/g) and ORL (6.27 log_{10} PFU/g) groups on D 1, one day after the second bacteriophage treatment and oral inoculation with *E. coli* O157:H7 (Figure 3-1A).

Populations then decreased throughout the experimental period, with the exception of a slight increase in phage levels on D 13 following the final phage treatment on D 9. By D 3, CON steers were shedding bacteriophages with the same plaque morphology as the administered phages in their feces at levels similar to REC steers ($P = 0.39$) even though phage was not directly administered to this group. Enrichment was required to detect phage in all treatment groups after D 20.

Furthermore, on D 3, plaques exhibiting a morphology and size that were different from that of the experimental phages (5.16 mm with concentric rings vs. 0.99 mm without rings) appeared on overlay plates of samples collected from O+R steers. By D 5, phages forming large plaques with this unique morphology were isolated from steers in all treatment groups. Though both small and large plaques could be found together on one plate, often one replicate plate would exhibit small plaques and the other replicate plate would exhibit large plaques (Figure 3-2A, B). Endogenous phage accounted for roughly 8 % of the total phage count.

Transmission electron microscopy of phages with this unique colony morphology indicated that they may to belong to the family (*Siphoviridae*), whereas inoculated phages were members of the *Myoviridae* family (Figure 3-2C, D, Table 3-2). Both families are members of the same order, *Caudovirales*. Subsequent phage counts during the study included both the administered and endemic phages. On this basis, mean treatment bacteriophage shedding levels (log PFU/g of feces) were higher ($P = 0.03$) in ORL and O+R than in CON or REC (Figure 3-1B). Bacteriophage-positive samples were 2.31 times more likely to be found in ORL ($P = 0.03$), and 1.91 times more likely to be found in O+R ($P = 0.07$), than in CON (Table 3-1).

All steers were culture positive for Nal^R *E. coli* O157:H7 on D 1 following inoculation on D 0 (Figure 3-3A). Mean treatment group shedding levels on D 1 ranged from 3.95 to 7.03 log CFU/g of feces. Nalidixic acid-resistant *E. coli* O157:H7 levels declined in all treatment groups until D 21, with the exception of a brief increase in shedding by CON, REC, and O+R steers on D 9, prior to the final phage treatment. Also, a rise in levels in the ORL and O+R groups occurred between D 27and 41. Thereafter, levels of Nal^R *E. coli* O157:H7 declined from D 49 until the completion of the experiment. The overall mean shedding level (log_{10} CFU/g of feces) was higher in REC steers ($P < 0.10$) than in the other three treatments (Figure 3-3B).

Figure 4A displays the fecal shedding patterns of Nal^R *E. coli* O157:H7 in individual animals throughout the trial. After D 13, individual animals often were culture-negative for a number of consecutive weeks before being once again confirmed positive for Nal^R *E. coli* O157:H7. Overall, the ORL steers had fewer ($P = 0.06$) Nal^R *E. coli* O157:H7 culture-positive samples compared to REC and O+R, but only nominally fewer ($P = 0.26$) than CON animals (Figure 3-4B).

Pulsed field gel electrophoresis analysis of the inoculated strains (Figure 3-5) revealed the anticipated differences in PFGE profiles that would enable assessment of the frequency of shedding of the five individual inoculated strains, and identification of any isolates with different PFGE profiles. Among 118 fecal or manure pat isolates (60 from D 1 and 58 from D 69, 76 and 83), 107 had the same PFGE profiles as the inoculated strains and 11 had different profiles. There was considerable variation in the frequency of the individual inoculated strains (Table 3-3). Nal^R *E. coli* O157:H7 strain 318N was the most frequent among the inoculated strains (48/107) both at the beginning and the end

of the trial, whereas strain E32511 was not among the isolates recovered. The others were intermediate in frequency (Table 3-3). The virulence of the inoculated phages for each of the five Nal^R *E. coli* O157:H7 strains included in the inoculum was quite similar (Table 3-4). Therefore, differences in the frequency of recovery of the different strains during the trial were not likely attributable to development of resistance to bacteriophages within specific strains.

The 11 isolates different from the inoculated strains by PFGE had five different PFGE profiles (Figure 3-5). All were isolated from fecal or manure pat samples collected late in the trial. Microplate assay analysis of the virulence of one strain representing each of two of the new PFGE profiles revealed that one had up to a 10^5 increase in resistance to phage wV8, though no such differences in resistance to the other three phages (rV5, wV7 and wV11) was evident (Table 3-4).

Both Nal^R *E. coli* O157:H7 and bacteriophage persisted in the environment for the majority of the 83 D trial. The O157-specific phages and Nal^R *E. coli* O157:H7 were isolated from pen floor manure samples up to D 76 and D 83, respectively. Bacteriophage and *E. coli* O157:H7 were found more frequently in manure samples than in water and feed samples (Table 3-5). Treatment did not have an effect on the prevalence of environmental manure samples culture-positive for *E. coli* O157:H7 ($P = 0.74$) or bacteriophage ($P = 0.56$).

3.4. DISCUSSION

Recent work has drawn attention to the recto-anal junction as the primary site of *E. coli* O157:H7 colonization in cattle (Naylor et al., 2007; Naylor et al., 2003). Sheng et al., (2006) demonstrated a reduction in fecal shedding of *E. coli* O157:H7 with rectal administration of bacteriophage (4 x 25 ml of 10^{10} PFU/ml). However, Sheng et al. (2006) also continuously administered bacteriophage orally via drinking water (1.8×10^6 to 5.4×10^6 PFU/ml), a factor that we believe to be the primary reason for the significant decrease in the shedding of *E. coli* O157:H7 observed in treated animals in their study. Our study examined both rectal and oral phage administration separately as well as in combination. We established that oral administration of bacteriophage resulted in fewer *E. coli* O157:H7-positive samples and a lower mean shedding level of *E. coli* O157:H7 than did rectal administration of bacteriophage. Colonization may be greatest at the recto-anal junction; however, *E. coli* O157:H7 has also been shown to colonize more proximal regions of the GIT (Grauke et al., 2002). Application of bacteriophage to the rectum would not facilitate distribution of phage to the upper regions of the GIT that could be colonized by *E. coli* O157:H7. Additionally, oral application of phage established a higher phage titre within the steers than did rectal application. This may be due to a longer period of retention of phage within the digestive tract as it flows with the liquid or solid phases of digesta. By increasing retention time and exposure to *E. coli* O157:H7 cells with oral application, bacteriophage likely have increased opportunity to interact with the target host and replicate, increasing their population and the likelihood of infection of additional *E. coli* O157:H7 cells. These factors likely contributed to the

more pronounced decrease in *E. coli* O157:H7 that was more pronounced in cattle orally treated with phage.

Though oral administration of bacteriophage significantly reduced the fecal shedding of *E. coli* O157:H7 by feedlot steers, *E. coli* O157:H7 was not eliminated. This finding is consistent with the only other known study to administer O157-specific bacteriophage to adult cattle (Sheng et al., 2006). Application of bacteriophage to sheep for the mitigation of *E. coli* O157:H7 was also not successful in eliminating the pathogen (Bach et al., 2003) though application to calves has resulted in complete elimination (Waddell et al., 2000). There are many reasons why it may not be possible to eliminate *E. coli* O157:H7 from mature ruminant animals. Phage particles must collide with a target host cell for infection and lysis to occur. As *E. coli* O157:H7 and bacteriophage populations decrease within the animal it becomes less likely that such a collision will occur within the voluminous ruminant digestive tract. This demonstrates a physical limitation of bacteriophage therapy. Further, if an animal does become culture negative, persistence of *E. coli* O157:H7 in the environment may result in re-coloniaztion. Our study demonstrated the persistence of *E. coli* O157:H7 in manure for the duration of the 12-week experimental period, though few positive samples were obtained from water and feed, as previously reported (Bach et al., 2005). Kudva et al., (1998) showed that *E. coli* O157:H7 survived in experimentally inoculated sheep manure, under fluctuating environmental conditions, for 21 months. Though it is known that shedding of *E. coli* O157:H7 in cattle is transient, it is possible that animals regaining culture-positive status after testing negative for a number of consecutive weeks may have been recolonized via the fecal-oral route (Stevenson et al., 2003).

This study employed a bacteriophage preparation with characteristics that in the past have previously proven successful in reducing target populations. The phage preparation consisted of a mixture of four phages (rV5, wV7, wV8, and wV11) and application of a "phage cocktail" has been shown to be more effective in reducing a target bacterial population than application of a single phage strain. Administration of multiple phage strains such as phage combinations SP21-22 (Tanji et al., 2004) and SP15-21-22 (Tanji et al., 2005) have been shown to slow the development of phage resistance in *E. coli* O157:H7 when compared to administration of a single strain. A phage cocktail may also attack a wider range of *E. coli* O157:H7 strains (Tanji et al., 2005).

It has also been shown that administration of multiple doses of a phage over a period of days is more effective than a single dose of phage, possibly aiding in the establishment of phage populations within the animal. Tanji et al. (2005) demonstrated that administration of a single oral dose of bacteriophage (10^8 or 10^{10} PFU) to mice did not alter *E. coli* O157:H7 levels, and was insufficient to establish a stable phage titre within the mouse GIT, while daily administration of phage (8 D experimental period) resulted in near elimination of *E. coli* O157:H7 in the mouse GIT and maintenance of a phage titre above 10^3 PFU/fecal pellet for 5 days. Sheng et al. (2006) also employed a multiple dosing regime by rectally administering phage to cattle four times as well as providing constant exposure with the inclusion of phage in water sources (maintained daily at 10^6 PFU/ml). In our study, phage titres in the ORL and O+R steers remained above 10^4 PFU/g feces for the first seven days following the initial phage administration (D -2), and above 10^2 PFU/g feces until at least D 13 of the trial. Two weeks after the

final phage administration, enrichment was required for the detection of bacteriophages in all treatment groups. Finally, success has also been shown when a phage preparation was administered both before and after bacterial challenge (Waddell et al, 2000). This may suggest that bacteriophage therapy works well as a preventative therapy as well as a treatment therapy (Smith and Huggins, 1982), especially if phages are present in the GIT prior to the establishment of *E. coli* O157:H7 colonization.

Development of phage-resistant mutants was noted early on in the history of bacteriohpage therapy and has continued to appear in recent work (Smith and Huggins, 1982; Tanji et al., 2005). A number of isolates collected at the end of this trial displayed PFGE banding patterns slightly different than that of the original five experimental strains. These isolates also demonstrated increased resistance to one of the four experimental phage strains. Smith and Huggins (1982) noted the presence of phage-resistant mutants in their *E. coli*/phage experiments; however, they chose to use a bacteriophage that was specific to a capsular antigen on the target cell involved in pathogenicity. This intuitive concept meant that if phage resistance developed, by loss or alteration of the target antigen, it would be reflected in the bacterial cell by a decline in virulence.

Counts of *E. coli* O157:H7 established using the challenge model in our study were much higher than those previously reported in naturally infected cattle (Omisakin et al., 2003; Zhao et al., 1995). However, animals shedding *E. coli* O157:H7 at nominal levels are not responsible for the majority of meat contamination during slaughter. A relatively small percentage of the herd (~9 %) consistently sheds very high levels of fecal O157 ($> 10^4$ CFU g^{-1} feces), with these animals being proposed to be responsible for

nearly 96 % of beef product adulteration (Omisakin et al., 2003). Bacteriophage therapy appears to be most effective in reducing *E. coli* O157:H7 levels when these levels are high (> 10^4 CFU g^{-1} feces). Therefore, bacteriophage administration could target the shedding of *E. coli* O157:H7 in the so-called super-shedders, significantly decreasing the incidence of meat adulteration and human illness.

In the past, bacteriophage therapy studies with ruminants have been carried out under experimental conditions unlike that of the commercial feedlot (Bach et al., 2003; Sheng et al., 2006). In such studies, animals receiving bacteriophage were housed in containment facilities, which prevented contact between animals within different treatments. This study deliberately examined the efficacy of bacteriophage therapy in a more typical production setting. Variables such as the environment and animal-to-animal contact were considered. Though two pens were designated for each phage treatment, steers in this study were permitted nose-to nose contact, and a water trough was shared by pens within the same treatment. In addition, each animal was also passed through a common handling area during treatment and sampling periods, in a manner that was similar to management practices that would be employed in a commercial feedlot. Under these conditions bacteriophages were present in the feces of CON steers on d 0, after only one application of phage to treatment cattle on D –2. Once in the CON steers, bacteriophage replicated to achieve levels that were not significantly different from those in the steers that were rectally treated with phage. Consequently, the possibility that the levels of bacteriophage in REC steers arose as a result of these animals acquiring phage from the environment cannot be eliminated. The fact that phage levels in the CON steers were similar to those in REC steers, clearly demonstrates that significant replication of

phage occurred within the digestive tract of CON steers. Environmental transmission of phage among cattle within the feedlot could be advantageous in reducing the levels of *E. coli* O157:H7 within the feedlot.

Transmission electron microscope images of phages isolated from large plaques appear to belong to the family *Siphoviridae,* due to the symmetrical cubic capsid head and long non-contractile tail, traits that are characteristic of this family (Ackermann, 2006). Many studies have examined *Siphoviridae* phage specific to *E. coli* O157 (Datz et al., 1996; Johansen et al., 2001; Tobe et al., 2006). A well-studied member of this family is bacteriophage lambda (λ), a temperate phage of *E. coli*. Phage of this nature are not of interest for bacteriophage therapy as they typically have a longer latent period and are more likely to transfer genes to the host through transduction (Datz et al., 1996). However, the quick spread of this phage from O+R steers (appearing first on d 3) to steers in the other treatment groups, further demonstrates the movement of phage through the environment and establishment of phage populations *in vivo*, via oral exposure.

Bacteriophages were found in pen-floor manure for a period up to D 76 of the trial. Manure samples positive for bacteriophage were not always positive for *E. coli* O157:H7, indicating survivability of phage in the absence of a detectable host. This environmental stability, in conjunction with the ability of phage to replicate *in vivo*, could be exploited for application on a larger scale, if bacteriophage could be administered with bedding or feed. Such a method of phage application would negate the need for individual administration to each animal and increase the likelihood of cattle acquiring phage on multiple days throughout the feeding period.

Bacteriophage therapy has been shown to be a successful mitigation strategy in controlling and treating various bacterial infections. In this study, the oral application of bacteriophage was more effective in establishing fecal phage populations and mitigating fecal *E. coli* O157:H7 in cattle than rectal bacteriophage application. The ability of phage to survive for extended periods in the environment, as well as the ability of phage to pass from one animal to another, suggests that large-scale application of phage to feedlot animals may be a feasible means of mitigating *E. coli* O157:H7 in the environment. Development of a method to administer phage in feed or water on a daily basis could make bacteriophage therapy a feasible tool for the mitigation of *E. coli* O157:H7 in feedlots.

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TABLE 3-1. *Odds ratios representing the likelihood of isolating bacteriophage or Nal^R Escherichia coli O157:H7 from fecal grab samples within a specified treatment group when compared to that of CON samples.*

Treatment	OR	95% CI	*P*-value
	Bacteriophage		
REC[1]	1.33	0.63 to 2.81	0.34
O+R[2]	1.91	0.92 to 3.98	0.07
ORL[3]	2.31	1.11 to 4.80	0.03
	Nal^R *Escherichia coli* O157		
REC[1]	1.51	0.62 to 3.72	0.27
O+R[2]	0.92	0.40 to 2.10	0.78
ORL[3]	0.72	0.32 to 1.60	0.31

[1]Rectal administration of 1.5 x 10^{11} PFU bacteriophage

[2]Oral and rectal administration of 4.8 x 10^{11} PFU bacteriophage total

[3]Oral administration of 3.3 x 10^{11} PFU bacteriophage

TABLE 3-2. *Capsid and tail measurements of the experimental phage wV7 and endogenous phage (large plaque-producing) as determined by transmission electron microscopy.*

Property (nm)	wV7	Endogenous
Capsid width	67.71	51.73
Capsid length	95.05	53.13
Tail width	92.64	158.67
Tail length	16.36	10.90

TABLE 3-3. *Numbers of fecal Nal^R* Escherichia coli *O157:H7 isolates collected[b] one day after experimental inoculation of steers or at the end of a 12-week experimental period that exhibited known or unknown PFGE banding patterns following* XbaI *digestion of genomic DNA*

Strain as identified by PFGE	CON		ORL		REC		O+R	
	d 1	End	d 1	End	d 1	End	d 1	End
R508N	4	1	2	2	2	3	2	0
E318N	8	6	4	10	4	8	5	4
H4420N	1	0	2	0	4	0	4	4
E32511	0	0	0	0	0	0	0	0
CO281-31N	4	0	6	6	4	1	4	2
Unknown[a]	0	2	0	1	0	6	0	2

[a] Banding patterns dissimilar to all of the five strains used in the experimental inoculation of steers.
[b] Isolated from fecal samples and environmental manure samples

TABLE 3-4. *Relative susceptibilities[a] of the five known strains of* Escherichia coli *O157:H7 and two novel isolates to each of the four bacteriophage strains used in the experimental cocktail*

	Bacteriophage			
E. coli O157:H7	rV5	wV7	wV8	wV11
Strain R508N	6	6	9	7
Strain E318N	8	5	8	7
Strain H4420N	6	6	5	7
Strain E32511	6	4	6	7
Strain CO281-31N	5	4	8	6
Isolate 492A	8	5	>10	7
Isolate 535A	6	6	9	7

[a] Values shown are lowest titres ($\log_{10}$ PFU/ml) at which complete lysis of *E. coli* O157:H7 occurred.

TABLE 3-5. *Numbers (and percentages) of positive* Escherichia coli *O157:H7 and O17-specific bacteriophage environmental samples collected over a 12-week experimental period.*

	Treatment				
Sample type[a]	CON	ORL	REC	O+R	Total
Water (n = 12)					
E. coli O157:H7	1 (8.3)	1 (8.3)	0 (0.0)	1 (8.3)	3 (6.3)
Bacteriophage	1 (8.3)	1 (8.3)	0 (0.0)	2 (16.7)	4 (8.3)
Feed (n = 24)					
E. coli O157:H7	2 (8.3)	1 (4.2)	2 (8.3)	2 (8.3)	7 (7.3)
Bacteriophage	0 (0.0)	3 (12.5)	0 (0.0)	1 (4.2)	4 (4.2)
Manure (n = 24)					
E. coli O157:H7	13 (54.2)	11 (45.8)	18 (75.0)	13 (54.2)	55 (57.3)
Bacteriophage	13 (54.2)	13 (54.2)	11 (45.8)	10 (41.7)	47 (49.0)

[a]Samples were collected weekly for 12 w. Samples were collected from each pen with the exception of water samples (100 ml), which were collected from troughs shared by pens within a treatment. Feed (10 g) and manure (1 g) sub-samples were taken from greater pooled samples for analysis.

Fig. 3-1

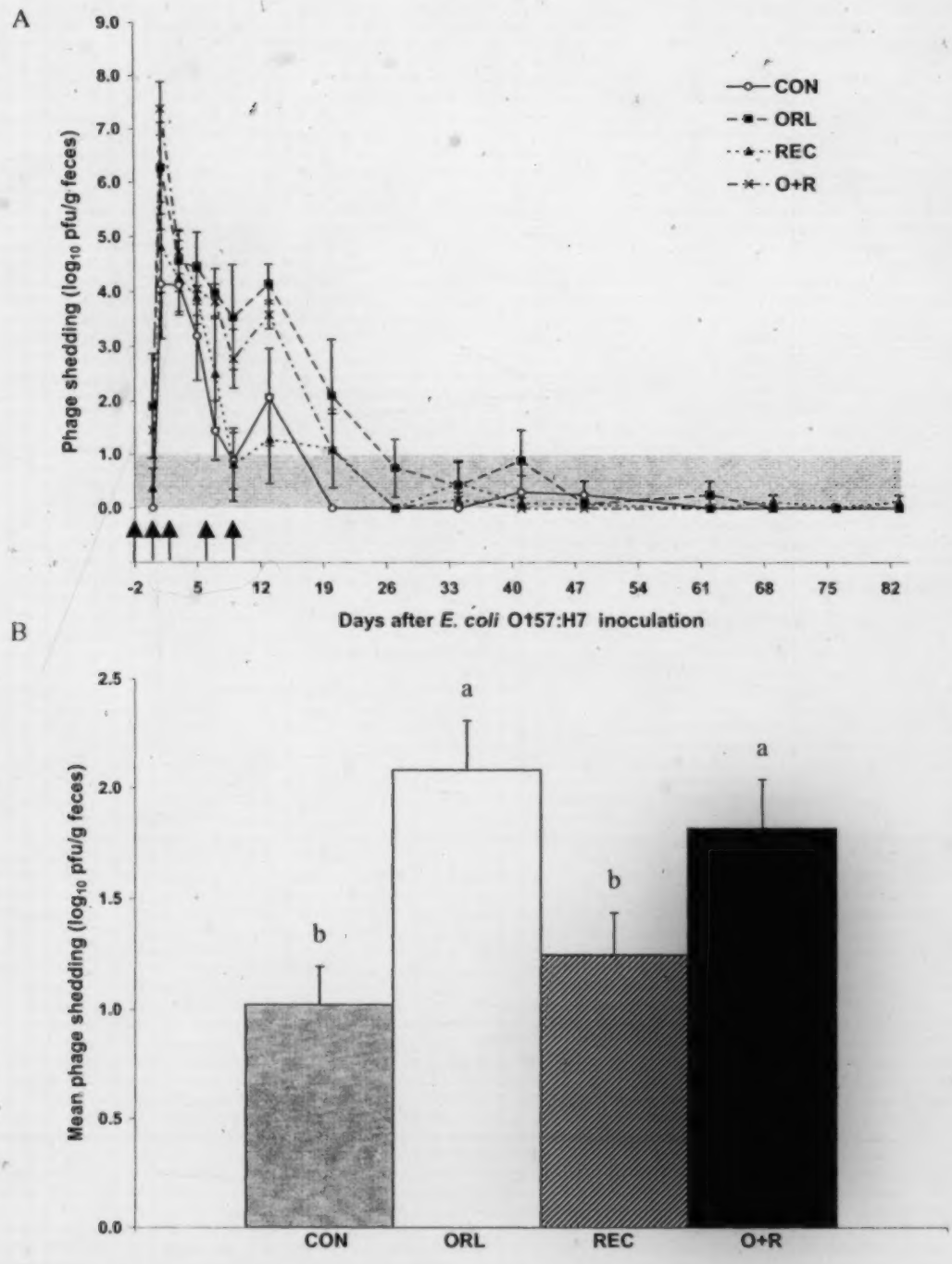

A
Phage shedding (log_{10} pfu/g feces)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
CON
ORL
REC
O+R
-2
5
12
19
26
33
40
47
54
61
68
75
82
Days after *E. coli* O157:H7 inoculation
B
Mean phage shedding (log_{10} pfu/g feces)
2.5
2.0
1.5
1.0
0.5
0.0
a
a
b
b
CON
ORL
REC
O+R

FIGURE 3-1. *Fecal shedding (A) and mean shedding levels (B) of bacteriophage by treatment in experimentally inoculated steers treated orally (ORL; 3.3 x 10^{11} PFU), rectally (REC; 1.5 x 10^{11} PFU), both orally and rectally (O+R; 4.8 x 10^{11} PFU), or not treated (CON) with O157-specific bacteriophage on days –2, 0, 2, 6, and 9. The shaded area in (A) represents shedding detected following enrichment. Day –2 data was not included in mean shedding levels as phage had not yet been administered at the time of sampling.* [a, b] *Bars with different letters are significantly different (P < 0.10).*

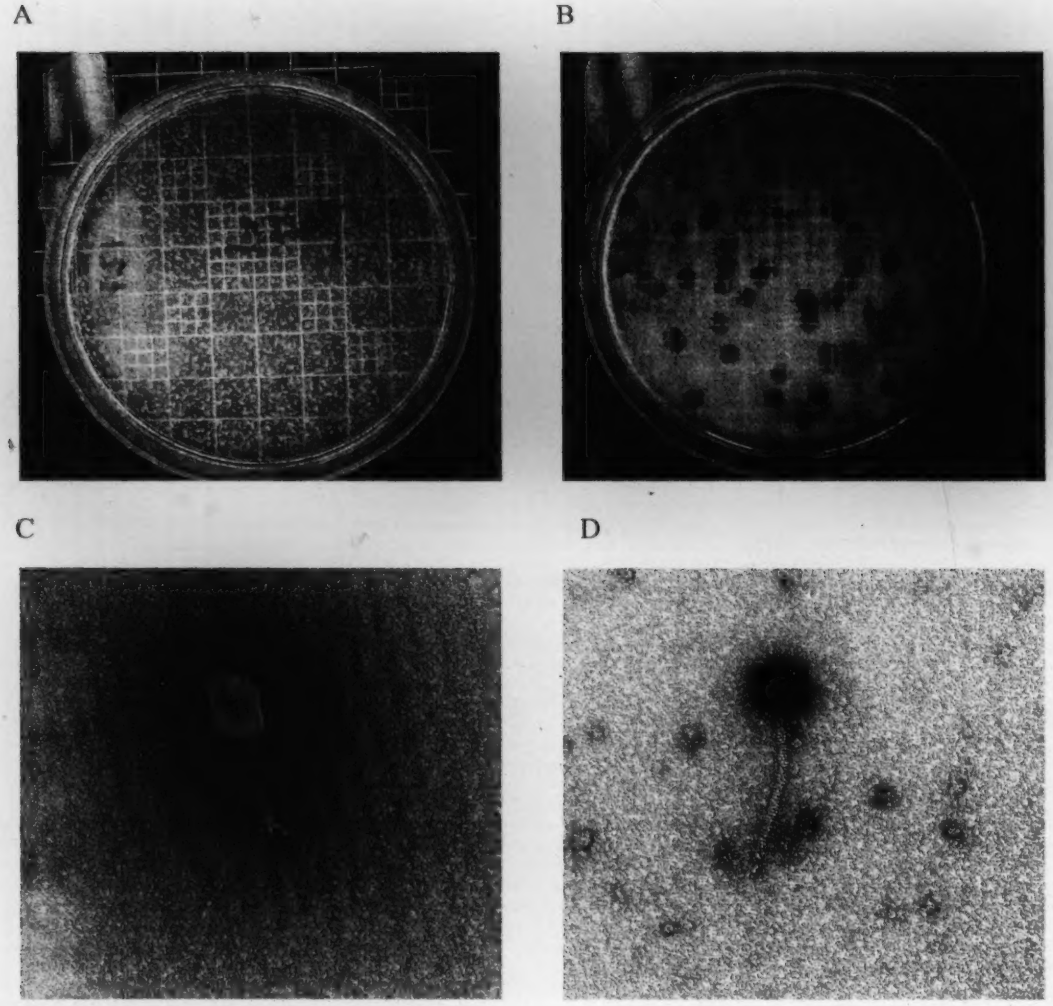
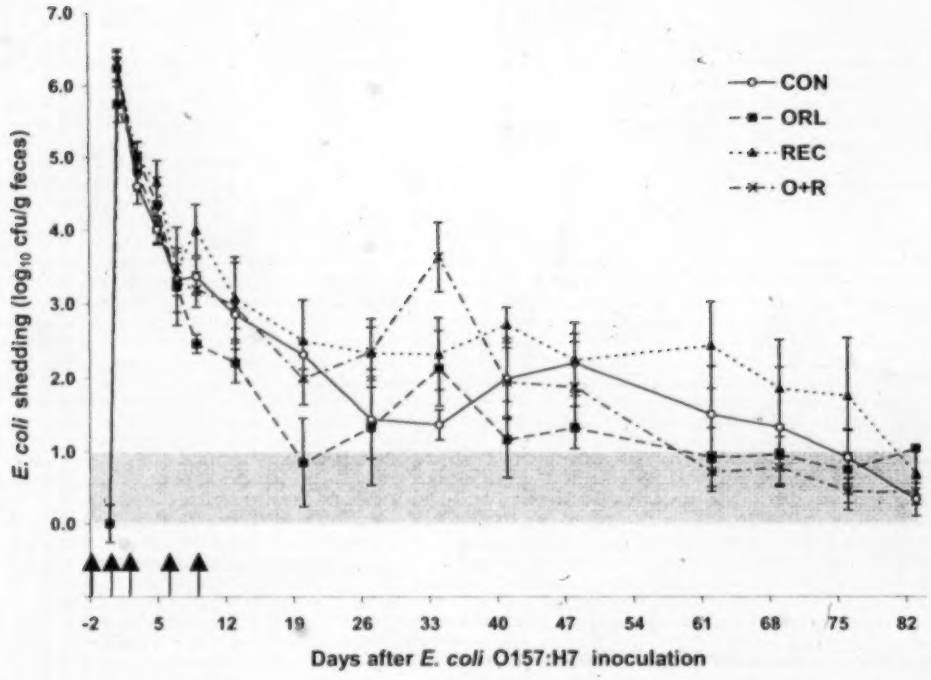


FIGURE 3-2. *Duplicate bacteriophage overlay plates showing the small plaques of the experimental phage (A) and large plaques (B) of the endogenous phage which was first isolated on d 3. Transmission electron microscope images of experimental phage wV7 (C) and the endogenous (large plaque-producing) phage (D).*

Fig. 3-3

A


7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
E. coli shedding (log10 cfu/g feces)
CON
ORL
REC
O+R
-2
5
12
19
26
33
40
47
54
61
68
75
82
Days after E. coli O157:H7 inoculation

B

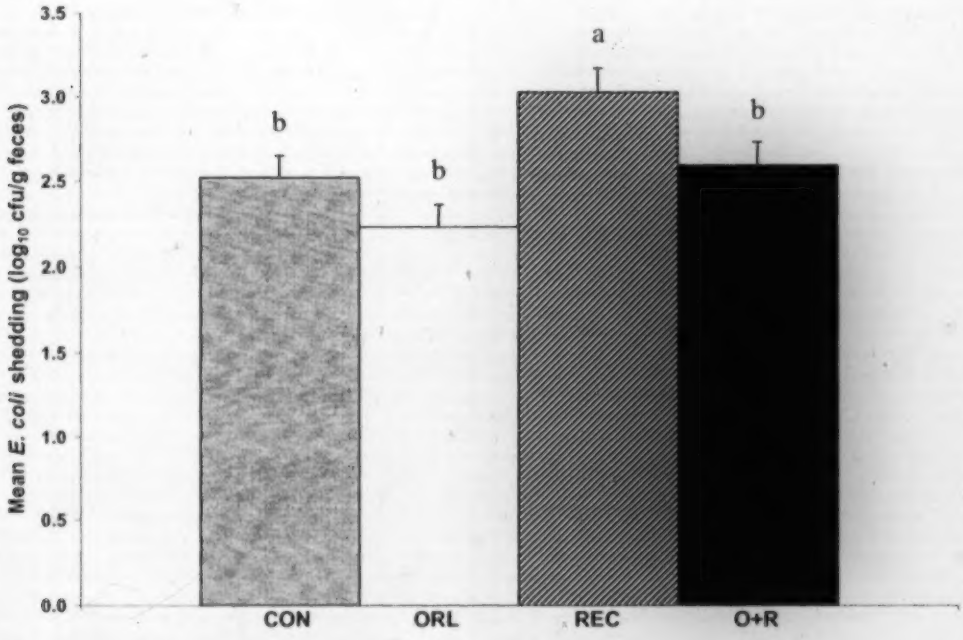
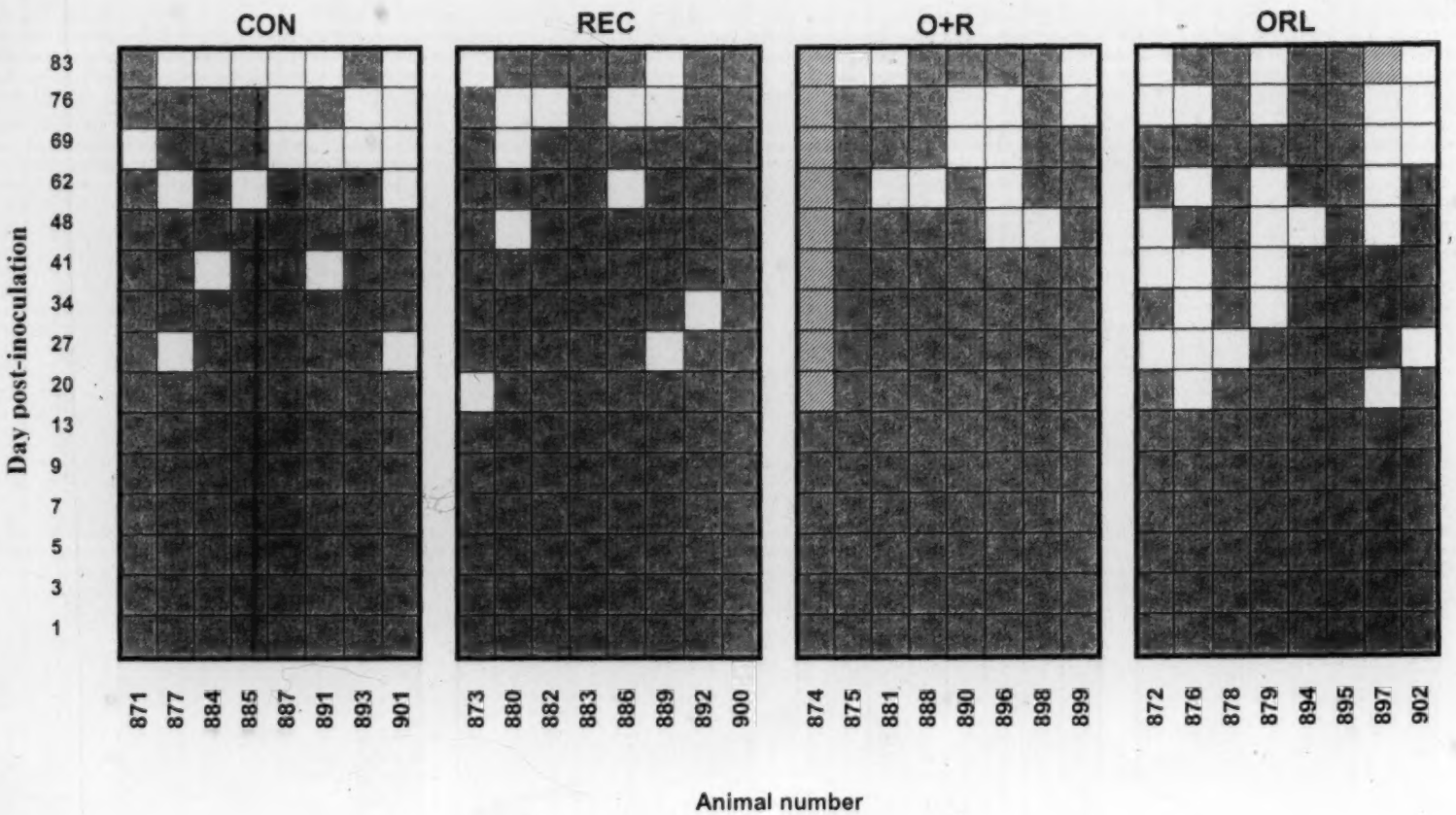

3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Mean E. coli shedding (log10 cfu/g feces)
a
b
b
b
CON
ORL
REC
O+R

FIGURE 3-3. *Fecal shedding (A) and mean shedding levels (B) of Nal^R E. coli O157:H7by treatment in experimentally inoculated steers treated orally (ORL; 3.3 x 10^{11} PFU), rectally (REC; 1.5 x 10^{11} PFU), both orally and rectally (O+R; 4.8 x 10^{11} PFU), or not treated (CON) with O157-specific bacteriophage on days –2, 0, 2, 6, and 9. The shaded area in (A) represents shedding detected following enrichment. Days –2 and 0 data was not included in mean shedding levels as Nal^R E. coli O157:H7 had not yet been administered at the time of sampling. $^{a, b}$Bars with different letters are significantly different (P < 0.10).*

Fig. 3-4

A


CON
REC
O+R
ORL
Day post-inoculation
83
76
69
62
48
41
34
27
20
13
9
7
5
3
1
871 877 884 885 887 891 893 901
873 880 882 883 886 889 892 900
874 875 881 888 890 896 898 899
872 876 878 879 894 895 897 902
Animal number

B

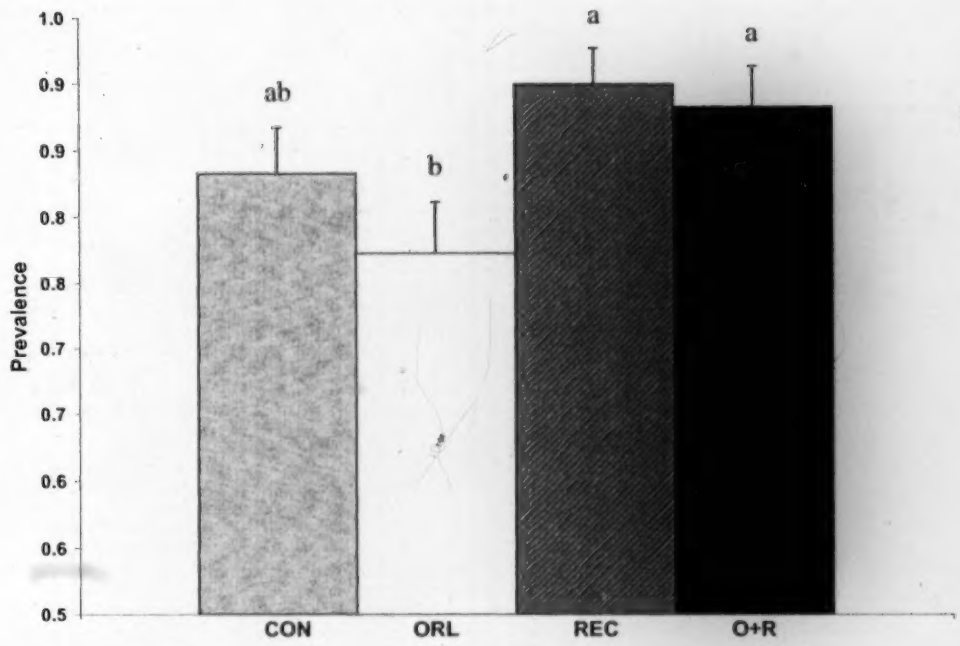


FIGURE 3-4. . *Fecal shedding patterns (A) and prevalence of culture-positive* E. coli *O157:H7 fecal samples (B) by animal from experimentally inoculated steers treated orally (ORL; 3.3 x 10^{11} PFU), rectally (REC; 1.5 x 10^{11} PFU), both orally and rectally (O+R; 4.8 x 10^{11} PFU), or not treated (CON) with O157-specific bacteriophage. In (A) grey cells represent* E. coli *O157:H7 culture-positive samples; white cells represent culture-negative samples. Hatched cells indicate no data available (early removal of animal 874).* $^{a, b}$*Bars with different letters are significantly different (P < 0.10).*

FIGURE 3-5. *Pulsed-field gel electrophoretic analysis of* XbaI*-digested genomic DNA from the five laboratory strains of* Escherichia coli *O157:H7 used to inoculate the steers and from two representative isolates that exhibited novel banding patterns. Lanes 1 to 5 contain the known* E. coli *O157:H7 strains E32511, H4420N, E318N, C0281-31N and R508N, respectively. Lane 6: Isolate 535B, obtained on d 83 from treatment group O+R; Lane 7 Isolate 492A (d 76; REC); Lanes labeled M contain molecular weight marker ranging from 48.5 to 485 kb.*

4. GENERAL CONCLUSIONS

4.1. Limitations

Thought there are many advantageous features of bacteriophage therapy, there are also limitations. These limitations occur as a result of the biological nature of phage therapy.

Early research demonstrated rapid clearing of administered bacteriophages from circulation when injected into mice and rats (Nungester and Watrous, 1934; Appelmans, 1921), with longer-term survival noted in the spleen of treated animals (Appelmans, 1921). In these studies bacteriophage was administered in the absence of a suitable host. A later study by Dubos et al., (1943) also showed a quick drop in circulatory levels of bacteriophage with interperitoneal administration in the absence of a host. However, when a *Shigella dysenteriae* preparation was injected intercerebrally along with interperitoneal phage administration, a high level of bacteriophage was noted in the brain of the mice. Also phage persisted in the circulatory system for ~75 – 138 h as long as phage levels remained high in the brain (Dubos et al., 1943). In these cases, inactivation of phage by antibodies (Merril et al., 2003) was not involved as clearance occurred much quicker than would allow the production of anti-phage antibodies. The reticuloendothelial system (RES) has since been identified responsible for the rapid clearance of phage from treated animals, which results in phage levels below that capable of clearing infection (Geier et al., (1973); Merril et al., 2003).

In an attempt to increase the period of circulation of administered phages, phage preparations were passed through experimental animals numerous times to generate a sub-population of "long-lasting" phages, possessing longer retention times in the circulatory system (Barrow & Soothill, 1997). The therapeutic value of these phages, however, was not determined (Barrow & Soothill, 1997).

Phage neutralizing antibodies have been isolated during bacteriophage therapy studies (Slopek & Ku..., 1987; Kucharewicz-Krukowska & Slopek, 1987). Antibodies appeared after a few weeks post-administration of phage and it is unclear for how long these antibodies would circulate or how quickly they would appear following additional rounds of phage administration. The production of *E. coli* O157-specific phage neutralizing antibodies in adult cattle has not yet been documented.

Administration of multiple doses of phage over a period of time appears to be more effective in mitigating the shedding of *E. coli* than does administration of a single dose. A treatment regime of multiple doses over time, however, presents the opportunity for clearing of phage by the reticuloendothelial system, as well as the potential development of antibodies against an administered phage preparation.

As noted above, one advantage of phage therapy is the specificity by which phages attack their host. Phage therapy can strategically eliminate a target pathogen without disturbing the natural microflora of the bovine GIT. This characteristic of phages, however, can also result in a limitation of phage therapy. Because phages are so specific, mutation in the host population may lead to decreased host susceptibility, or increased resistance, to the therapeutic phage. Resistance of *E. coli* to bacteriophage has been documented in phage therapy studies (Smith and Huggins, 1982; Tanji et al., 2005).

Another limitation resulting from phage specificity is the difference in susceptibility among various *E. coli* O157 strains. The distribution of *E. coli* O157:H7 strains are regional; therefore a phage cocktail that is successful in mitigating *E. coli* O157:H7 shedding in one herd or region may not be appropriate for another. The use of a phage cocktail as opposed to a single strain of phage has been suggested to combat the limitations resulting from phage specificity (Tanji et al., 2005).

4.2. General Conclusions

Cattle are considered an important reservoir of the human pathogen *Escherichia coli* O157:H7 (Hancock et al., 2001; Renter and Sargeant, 2002) and fecal shedding of *E. coli* O157:H7 by cattle has been associated with contamination of beef products (Elder et al., 2000). For the past 15 years, researchers have investigated various methods for mitigating *E. coli* O157:H7 in cattle, though at this time no one method offers an exclusive solution to this problem. This thesis attempted to further our knowledge in the use of bacteriophage therapy for controlling *E. coli* O157:H7 in cattle by comparing current methods of phage administration and presenting an opportunity for future large-scale application. Chapter 2 highlighted the importance of *E. coli* O157:H7 as a foodborne pathogen and the association of this pathogen with cattle. Chapter 2 also discussed the various approaches that have been examined for *E. coli* O157:H7 mitigation. Chapter 3 compared oral and rectal application of a bacteriophage cocktail to experimentally inoculated steers and monitored bacteriophage in the environment of these steers.

Prior to completing the research presented in this thesis it was known that bacteriophage therapy could reduce the fecal shedding of *E. coli* O157:H7 in cattle, as discussed in Chapter 2. Recently, the recto-anal junction was identified as an important region of *E. coli* O157:H7 colonization in cattle (Naylor et al., 2003) and subsequent mitigation studies have targeted this area of the animal using phage therapy (Sheng et al., 2006). In fact, the work conducted in my thesis was in progress when the manuscript of Sheng et al. (2003) was published in which similar phage-based strategies were used in an attempt to control *E coli* O157:H7. In Chapter 3, we showed that oral administration of bacteriophage was superior to rectal administration of bacteriophage in a number of ways. Oral treatment resulted in a higher mean shedding level of bacteriophage than did rectal treatment. As discussed, a higher shedding level was likely due to a longer retention time of the phage within the steers, resulting in a longer period of phage replication prior to excretion with digesta. Oral phage treatment also resulted in a lower mean shedding level of *E. coli* O157:H7. The research presented in Chapter 3 indicates that, though a high proportion of *E. coli* O157:H7 colonization occurs at the recto-anal junction, oral application of bacteriophage is more effective in controlling *E. coli* O157:H7 in cattle than rectal application of a bacteriophage cocktail.

Current rectal and oral administration strategies require multiple treatments with phage. Such a treatment strategy would require a large investment of time, especially in a facility such as a feedlot. It would therefore be advantageous to develop a large-scale method of phage administration. In Chapter 3, bacteriophages were found in the manure on the pen floor in pens housing steers that had been treated with bacteriophage. It is unknown, however, how long viable phage populations could last under various

environmental conditions. A possible next step in bacteriophage therapy research would be to develop phage preparations that were inert in the environment, but remained infective once they entered the GIT of an animal, giving them the opportunity to interact with host cells. In Chapter 3, steers that had not been treated with bacteriophage appeared to acquire phage from their environment, likely from the manure of phage-treated steers present in common areas, such as the chute and squeeze. Ingested bacteriophages were then able to replicate within the animals. Development of an environmental phage delivery protocol may make bacteriophage control of *E. coli* O157 more efficacious in a commercial feedlot.

Bacteriophage therapy has been successful in treating bacterial infections in mice (Tanji et al., 2005; Sheng et al., 2006; Smith and Huggins, 1982) and even infections with *Shigella, Staphylococcus, Pseudomonas* and *E. coli* in humans (Sulakvelidze et al., 2001). With the exception of one study (Waddell et al., 2000) bacteriophage therapy has not completely eliminated *E. coli* O157:H7 from inoculated steers. In mice and human studies, bacteriophage administration was employed to treat bacteria that caused disease in their host, however, this is not the case with *E. coli* O157:H7 and cattle. *E. coli* O157:H7 is not a bovine pathogen and naturally colonizes healthy cattle (Montenegro et al., 1990). Though bacteriophage therapy can clear bacterial infections in humans and mice, the lack of an immune response towards *E. coli* O157:H7 in cattle may explain why phage therapy has not been able to eliminate *E. coli* O157:H7 from cattle. An immune response against *E. coli* O157:H7 may help to target cells when present at low levels, when the rate of bacteriophage-host collision is also low. It may be necessary to combine phage therapy with another method of mitigation, such as the immunization strategy

outlined by Potter et al., (2004), in order to completely eliminate *E. coli* O157:H7 from cattle.

An important step towards implementing bacteriophage therapy as an *E. coli* O157:H7 mitigation strategy in cattle is ensuring human safety in the case of O157-specific bacteriophage consumption. Bacteriophages are highly specific to their target host cell and would most likely present no danger if consumed along with beef products. Bacteriophages have even been isolated from human stool samples (Chibani-Chennoufi et al., 2004). A recent study administered *E. coli*-specific bacteriophage T4 to healthy adults and no adverse effects were noted (Bruttin and Brüssow, 2005).

The number of cases of human *E. coli* O157:H7 infections have increased over the past 3 years. Because beef products are often implicated in foodborne outbreaks and because *E. coli* O157:H7 colonization of cattle is correlated with adulteration of beef products, control of this human pathogen in cattle must be addressed. Bacteriophage therapy shows promise as an efficacious mitigation strategy in cattle due to the high specificity and environmental persistence of bacteriophages and the possibilities of large-scale application.

4.3. References

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